Vol. XLIV No. 10

October 1955

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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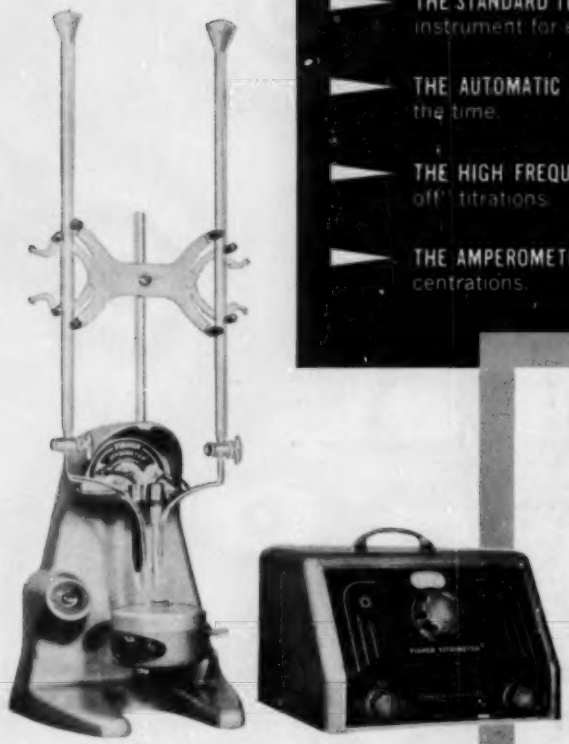

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1956 Iodine Research Award Nominations Requested Prior to January 1, 1956

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1956 Chilean Iodine Educational Bureau, Inc. Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1955 Award, *nominations must be received on or before January 1, 1956.*

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke; Paul Block, E. A. Brecht, Stanley G. Mittelstaedt; Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its eighth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Veron H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award which was conferred during the 1955 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

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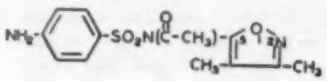
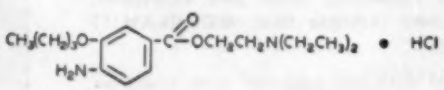
ACETYL SULFISOXAZOLE.—Gantrisin Acetyl.—$C_{13}H_{15}N_3O_4S$.—M.W. 309.35.—N^1-(Acetyl-3,4-dimethyl-5-isoxazolyl)sulfanilamide.—The structural formula of acetyl sulfisoxazole may be represented as follows:



Actions and Uses.—Acetyl sulfisoxazole shares the actions and uses of the parent sulfonamide, sulfisoxazole (Gantrisin). (See New and Nonofficial Remedies 1955, under the monograph on sulfisoxazole.) The acetyl derivative is tasteless and therefore suitable for oral administration, especially in liquid preparations of the drug. There is evidence to support the assumption that the acetyl compound is split in the intestinal tract and absorbed as sulfisoxazole; hence, the absorption, excretion, and solubility of acetyl sulfisoxazole in body fluids are considered to be the same as for the parent drug. It has been found to have about the same toxicity as sulfisoxazole and should be employed with the usual precautions for sulfonamide compounds.

Dosage.—Acetyl sulfisoxazole is administered orally. The dosage is expressed in terms of sulfisoxazole and calculated on the basis of 0.5 Gm. per 20 lb. (9 Kg.) of body weight as the initial dose, followed by one-half the initial dose every 4 hours. In severe infections, these doses may be doubled.

BENOXINATE HYDROCHLORIDE.—Dorsacaine Hydrochloride.—$C_{17}H_{28}N_2O_3 \cdot HCl$.—M.W. 344.88.—β-Diethylaminoethyl 4-amino-3-*n*-butoxybenzoate hydrochloride.—The structural formula of benoxinate hydrochloride may be represented as follows:



Actions and Uses.—Benoxinate hydrochloride, a benzoic acid ester related to procaine, is an effective surface anesthetic agent useful in ophthalmology for tonometry, gonioscopy, removal of corneal foreign bodies, and for short operative procedures involving the cornea and conjunctiva. It also has bacteriostatic properties. When applied locally to the conjunctiva and cornea, it produces slightly more intense anesthetic effect and is less irritating to the conjunctiva than comparable concentrations of tetracaine hydrochloride. A single instillation of 0.08 cc. of a 0.4% solution produces, within 60 seconds, a sufficient degree of anesthesia to permit tonometry or, after three drops at 90-seconds intervals, removal of a foreign body embedded in the corneal epithelium; however, a decrease in the depth of anesthesia is noted after 20 to 30 minutes, and the sensitivity of the cornea returns to normal within one hour. This relatively short duration of anesthesia reduces the risk of exposure keratitis in minor procedures not requiring an eye bandage. The same instillation produces little conjunctival irritation; in most patients there is no visible hyperemia, increased winking, or lacrimation. Instillations up to 0.5 cc. of a 0.4% solution do not produce any measurable alteration in the size of the pupil or its reaction to light, nor is accommodation affected. Large single doses of 1 cc. of the 0.4% concentration do not produce symptoms suggestive of systemic action.

Benoxinate hydrochloride and tetracaine have about the same toxicity index when compared with cocaine, given as an intravenous injection in experimental animals. Clinically, no signs of local or systemic hypersensitivity have followed its prolonged use in the eye; it has been tolerated by some patients with a history of sensitivity to other commonly employed local anesthetic agents. Nevertheless, it should be employed with the usual precautions for surface anesthesia and should be used sparingly in patients with known allergies, cardiac disease, hyperthyroidism, or open lesions.

Dosage.—Benoxinate hydrochloride is administered only by topical instillation in the eye. Two drops of a 0.4% solution, well instilled, is usually adequate for tonometry; a third drop invariably permits measurement of ocular tension and insertion of a contact lens without delay. Within 4 to 5 minutes, three two-drop instillations at 90-second intervals usually insure adequate surface anesthesia for removal of an embedded foreign body in the cornea or for opening a chalazion through the conjunctival surface.

CARBINOXAMINE MALEATE.—Clistin Maleate.—$C_{16}H_{19}ClN_2O \cdot C_4H_4O_4$.—M.W. 406.88.—2-[*p*-Chloro - α - (2 - dimethylaminoethoxy)benzyl]-pyridine maleate.—The structural formula of carbinoxamine maleate may be represented as follows:

Actions and Uses.—See New and Nonofficial Remedies 1955, under the general statement on histamine-antagonizing agents. Carbinoxamine maleate has as potent antihistamine action and as low an incidence of side-effects as has any other previously employed histamine antagonist. At its antihistamine level of action the drug exhibits comparatively weak atropine-like anticholinergic

(Continued on page VI)

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 200 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

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(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

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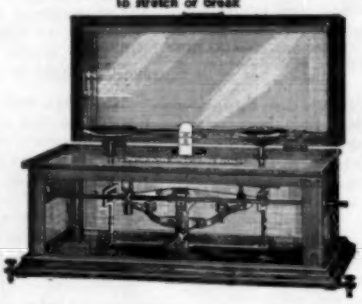

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. . . . . *from page IV*

activity or ganglionic blockade in experimental animals, and it is not likely to produce cardiovascular or respiratory manifestations of such effects in human beings. It does not potentiate epinephrine or exhibit local anesthetic action.

Dosage.—Carbinoxamine maleate is given orally. The usual effective dose for adults is 4 mg., three to four times daily. Larger doses of 6 to 8 mg. usually are tolerated if needed to produce the desired antihistaminic effect. Children over 6 years of age usually respond to oral doses of 2 mg., three to four times daily; smaller doses may be required for younger children.

ERYTHROMYCIN GLUCOHEPTONATE.—Ilotycin Glucoheptonate.—Erythromycin glucoheptonate is the glucoheptonate salt of erythromycin, an antibacterial substance produced by the growth of *Streptomyces erythreus* Waksman. The structural formula of erythromycin glucoheptonate has not been established.

Actions and Uses.—Erythromycin glucoheptonate has the same action and uses as the base except that it is primarily suited for intravenous injection. (See New and Nonofficial Remedies 1955, under the monograph on erythromycin.) Perenteral administration is indicated for erythromycin-susceptible infections in patients who are unconscious, who are vomiting, or who are unable for any other reason to take the drug orally. In such cases oral medication with the base or other salts should be resumed as soon as the patient can ingest and retain it.

Dosage.—For intravenous injection, an initial solution should be prepared by completely dissolving the equivalent of 0.25 Gm. of erythromycin base in not less than 10 cc. of water for injection, U. S. P. This solution retains its potency for 7 days if kept in a refrigerator. *Saline or other diluent should not be employed for making the initial solution in order to avoid gel formation or slow and incomplete solution of the drug.*

For adults, the initial solution should be added to 250 to 500 cc. of isotonic sodium chloride solution or 5% dextrose solution and administered by slow intravenous infusion for 20 to 60 minutes. This dose (0.25 Gm. equivalent to the base) may be repeated every 6 hours. A continuous slow infusion, administering the equivalent of 1 to 2 Gm. of the base over a 24-hour period, may be employed as an alternate method.

For children, the initial solution is diluted as for adults but is administered in doses calculated on the basis of the equivalent of 5 mg. of the base per pound (about 11 mg. per Kg.) of body weight every 6 hours. Although the initial solution may be used for intravenous injection over a 5-minute period without severe reactions, this is not recommended because of nausea, vomiting, or pain along the vein; thrombosis has occurred in some instances.

(Continued on page VII)

N. N. R.

. . . . . from page VI

ERYTHROMYCIN LACTOBIONATE.—Erythrocin Lactobionate.—Erythromycin lactobionate is the lactobionate salt of erythromycin, an antibacterial substance produced by the growth of *Streptomyces erythrus* Waksman. The structural formula of erythromycin lactobionate has not been established.

Actions and Uses.—Erythromycin lactobionate, a water-soluble salt of erythromycin suitable for intravenous or intramuscular injection, has the same actions and uses as the base. (See New and Nonofficial Remedies 1955, under monograph on erythromycin.) Injection of the drug is indicated in patients unable to tolerate oral medication or in whom high blood levels are necessary to control severe erythromycin-susceptible infections resistant to other antibiotics. Oral therapy with erythromycin base or other salts should be resumed as soon as this becomes feasible. Erythromycin lactobionate, injected properly, has not been reported to produce thrombophlebitis following intravenous administration or tissue damage following intramuscular injection, but moderate to severe pain usually accompanies the latter.

Dosage.—Erythromycin lactobionate is administered either intravenously or intramuscularly, preferably by the former route to avoid pain produced by the latter route. Dosage is expressed in terms of erythromycin base. The usual dosage for children and adults is 1 to 2 mg. per lb. (2.2 to 4.4 mg. per Kg.) of body weight either intravenously or intramuscularly, injected at intervals of 8 to 12 hours.

A 5% "stock" solution (equivalent to 50 mg. of erythromycin base per cubic centimeter) first should be prepared by completely dissolving the equivalent of 0.3 Gm. of erythromycin base in 6 cc. (or the equivalent of 1 Gm. of base in 20 cc.) of either water for injection or 5% dextrose solution. *Isotonic sodium chloride solution (normal saline) or other inorganic salt solutions should never be used as a solvent for preparing the 5% "stock" solution of erythromycin lactobionate because they cause precipitation of the active ingredient at that concentration.* In the dry form the salt is stable at ordinary temperatures for prolonged periods; the 5% solution is stable for 2 weeks when stored in a refrigerator.

For intravenous injection, the 5% solution should be diluted with not less than four volumes of either 5% dextrose or isotonic sodium chloride solution to make a final concentration of not more than 1% (equivalent to 10 mg. of erythromycin base per cubic centimeter). The calculated dose should be injected slowly over a period of not less than 5 minutes to avoid pain along the course of the vein; alternatively, the calculated intravenous dose may be administered by infusion by diluting this in 200 to 500 cc. of 5% dextrose or isotonic sodium chloride solution.

For intramuscular injection, the 5% "stock" solution is used undiluted. The calculated dose should be injected deeply into a large muscle with extreme care to avoid subcutaneous deposition. The pain following intramuscular injection is caused by transitory local irritation. A single intramuscular dose should not exceed 0.5 Gm., as a precaution against the production of tissue damage.

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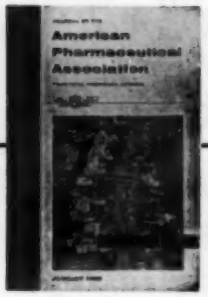

A Preview of Advertising

In the October 1955 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ **Abbott's** sales acumen and awareness of *media-impact* are reflected in its reservation of *four* pages to publicize its antibiotic, **Erythrocin; Iberol,** *the Iron-Plus formula,* and **Sur-Bex** *with* **C,** its potent dietary supplement.

▸ *Savings to you* are emphasized by the **American Druggists' Insurance Co.,** whose policies are available *only* to pharmacists.

▸ **The Ames Company's Bischoff Division** is on stage with **Aminet,** *for rapid relief in asthma,* and **Diatussin,** *drop dosage cough control.*

▸ Available through wholesalers or the Colgate-Palmolive Co. is a *new one inch size* **Vaseline** gauze strip manufactured by the *Professional Products Division* of the **Chesebrough Mfg. Co.**

▸ The "anesthetic-antiseptic" efficacy of its **Thantis Lozenges** is re-emphasized by **Hynson, Westcott & Dunning.**

▸ Interested in handling new pharmaceutical products throughout eastern Canada is **Lancet, Ltd.** of Québec, manufacturers' representatives.

▸ Marketed by **Lederle Laboratories** are **Incremin Drops,** described as a palatable liquid for stimulating infants' appetites and growth.

▸ To **V-Cillin, Lilly** ascribes higher blood levels, produced *faster* and maintained *longer.*

▸ Published by **J. B. Lippincott Company** and publicized in these pages is the **U. S. Dispensatory.**

▸ **Salcort,** *a new antiarthritic,* offered by **The S. E. Massengill Company,** is described as being "virtually without side effects."

▸ "A-1 Booster for Your B-Complex Sales" is the **Parke, Davis** announcement to pharmacists urging them to keep **Combex** products stocked.

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▸ "*When hormones are preferred therapy . . .* **Schering Hormones** *assure superior quality,*" according to their October announcement.

▸ *Three* potent antibiotics, *plus* a topical anesthetic, account, says **Sharp & Dohme,** for **Tetrazets'** usefulness "in sore-throat weather."

▸ The *new* **Benzedrex Inhaler** *display carton,* says **Smith, Kline & French,** will "double your . . . sales."

▸ To its prenatal supplement, **Calcisalin, Warner-Chilcott** adds the tag-line—*for a better pregnancy.*

▸ **Pediatric Neo-Synephrine** nasal spray for children is advocated by **Winthrop-Stearns** for the relief of nasal congestion in children's colds, sinusitis and hay fever.

▸ **Wyeth** announces a *new* antibiotic, **Pen-Vee-*Oral,*** for oral administration in streptococcal, pneumococcal, gonococcal and sensitive staphyloccal infections.

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out October 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *OCTOBER 1955* — NUMBER 10 — CONSECUTIVE NO. 20

The Synthesis of Some 4-Carbobenzoxy-3-alkoxy Benzamidine Hydrochlorides and Other Amidines*

By PAUL G. BAY† and OLE GISVOLD

This paper deals with the preparation of some 4-carbobenzoxy-3-alkoxy benzamidines, N-benzyl *p*-N-*n*-alkylamidine benzamides and benzyl *p*-N-*n*-butyl amidine thiobenzoate. It is hoped that these compounds can be screened for their anesthetic activities in order that they may be correlated with the marked anesthetic activities of some previously described esters of *p*-carboxybenzamidine.

A SERIES of esters of *p*-carboxybenzamidines some of which contain an alkyl substituted amidine group have been prepared in this laboratory (1–3). Some of these compounds were considerably more active than cocaine when instilled in guinea pig eyes and procaine when administered intracutaneously in guinea pig skin.

A series of N-alkyl *p*-amidobenzamidines also has been prepared (4) in order to compare the biological activity of the amide group versus the ester group. These compounds are at present being screened for their activities.

Recently Clinton and co-workers (5, 6) have reported the preparation of a number of *ortho*-alkoxy *p*-aminobenzoic acid esters. These *ortho*-alkoxy *p*-aminobenzoic acid esters exhibited considerably greater anesthetic activity as compared to their parent compounds. It also has been observed that in some cases the conversion of the ester linkage in certain local anesthetics to an amide linkage resulted in a moderate to a large increase in anesthetic activity (7). The replacement of the ether oxygen by sulfur in some anesthetics exerted a favorable effect (8). Thus members of a series of esters of *p*-amino thiobenzoic acid containing an *ortho*-alkoxy group exhibited more potent anesthetic activity than any of the analogous esters or amides (8). The toxicity ratios also were favorable.

It seemed desirable to prepare structurally related compounds in the previously described amidine series (1–3) of which the two most active were benzyl *p*-N-*n*-butyl amidino benzoate hydrochloride and benzyl *p*-amidino benzoate hydrochloride. Therefore, the benzyl group was retained in the preparation of some 4-carbobenzoxy-3-alkoxy benzamidines, N-benzyl *p*-N-*n*-alkyl amidino benzamides and benzyl *p*-N-*n*-butyl amidino thiobenzoate.

The experimental part of this paper deals with the preparation of a series of 4-carbobenzoxy-3-alkoxy benzamidine hydrochlorides, the preparation of a series of N-benzyl *p*-N′-*n*-alkylamidino benzamide hydrochlorides, and the preparation of benzyl *p*-N-*n*-butylamidino thiobenzoate hydrochloride. Part II of this paper also introduces a new method for the preparation of alkyl substituted amidines.

The results of the pharmacological tests of these new amidines will enable a direct comparison with the existing analogs. It will be possible to study the effect of the introduction of an *ortho*-alkoxy substituent in esters of *p*-carboxybenzamidines which previously have been prepared. It will

* Received April 28, 1955, from the University of Minnesota College of Pharmacy, Minneapolis 14.

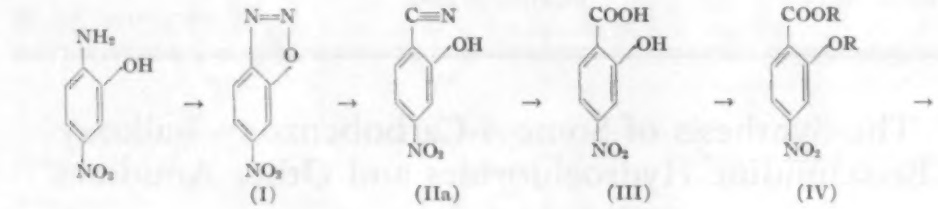
Abstracted from a dissertation submitted to the Graduate School of the University of Minnesota by Paul G. Bay in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

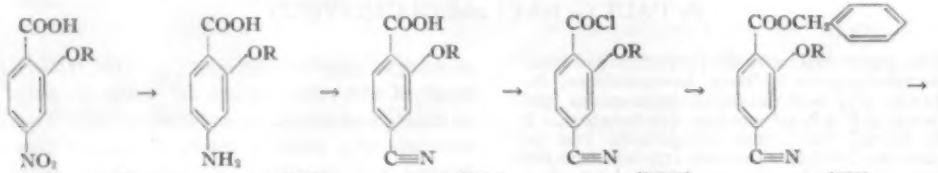
† Fellow of the American Foundation for Pharmaceutical Education, 1952–54.

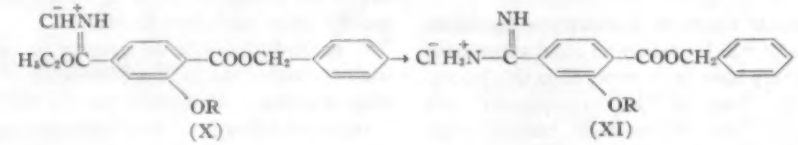
also be possible to observe the change in activity resulting from the replacement of an ester-linkage for an amide linkage in the known benzyl *p*-N-*n*-alkylamidino benzoate hydrochlorides. In addition the effect of the replacement of a thioester linkage for an ester linkage in benzyl *p*-N-*n*-butylamidino benzoate hydrochloride will be made apparent.

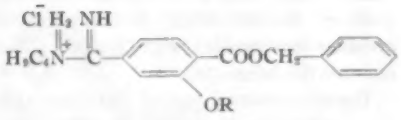
EXPERIMENTAL (PART I)

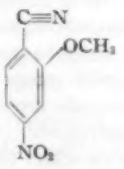
The following reactions serve to illustrate the sequence of reactions that are described in this experimental part.











4-Nitro Benzene Diazoxide[1] (I).—This starting compound was prepared from 2-amino-5-nitrophenol essentially according to the method of Clinton and Laskowski (9). When a quantity of the phenol as small as 0.1 mole was used, 125 cc. of water was necessary to solubilize it in dilute hydrochloric acid. This quantity of water was considerably larger in proportion to that reported by the above researchers, but was found necessary to equal the reported yields.

2-Hydroxy-4-nitro Benzonitrile (IIa).—This intermediate also was prepared according to a method described in the above report[2] (9).

2-Methoxy-4-nitro Benzonitrile (IIb).—Eight and four-tenths grams of 2-methoxy-4-nitro aniline[3] was placed in a solution of 20 cc. of water and 20 cc. of concentrated hydrochloric acid, and the temperature brought from 0 to 5°. A solution of 7.0 Gm. of sodium nitrite dissolved in 20.0 cc. of water was then cooled to 0° to 5° and added slowly with stirring, to the above mixture. After completion of the reaction 16.0 Gm. of sodium carbonate monohydrate was added. This solution was then added slowly, with stirring to a 60-cc. solution of 9.2 Gm. of sodium cyanide and heated on a steam pot until foaming ceased. Excessive foaming was controlled by the addition of small quantities of ether. The heating was then continued for an additional thirty minutes. The compound that separated was recovered, decolorized by boiling for ten minutes with ethyl acetate and 10 Gm. of carbon. A yield of 3.3 Gm. (37.0%), m. p. 175–177° was thus obtained (reported value 180°). This method was

[1] We are indebted to Mr. Waldbauer at General Aniline and Film Corp. for supplying us with this starting compound.

[2] The authors wish to acknowledge the generous assistance provided by Dr. Suter and Dr. R. O. Clinton at Sterling-Winthrop Research Institute in submitting to us a goodly supply of this intermediate.

[3] This compound was purchased from Eastman Kodak Co.

impractical when the amount of starting material approached molar quantities, for the yields then became negligible.

***p*-Nitro Salicylic Acid (III).**—This intermediate was obtained from either 2-hydroxy-4-nitro benzonitrile (IIa) or the corresponding methoxy derivative (IIb), by hydrolysis with sulfuric acid according to the method of Clinton and co-workers (10).

Alkyl 2-Alkoxy-4-nitro Benzoates (IV) and the Corresponding 2-Alkoxy-4-nitro Benzoic Acids (V).—These materials also were prepared according to methods reported in the literature (4).

2-Alkoxy-4-amino Benzoic Acids (VI).—In the preparation of these compounds, 500 mg. of platinum oxide was employed for each 0.9 mole of nitro compound. The solvent used was absolute ethanol. The reductions were conducted at 25° in a Parr low pressure hydrogenator applying an initial pressure of four atmospheres. The reaction times used were 0.25, 1.0, 14.0 and 12.0 hours respectively for the methoxy, ethoxy, propoxy, and butoxy analogs. The reaction mixture was heated if necessary to effect solution before removing the platinum oxide. The final compound was then obtained by crystallization from dilute ethanol.

2-Alkoxy-4-cyano Benzoic Acids (VII).—The method reported by Miller and Gisvold (11) was employed in the synthesis of these intermediates with the following important modifications. The reported amounts of acid, ice, and water were increased approximately 3.5 times to minimize intermolecular reaction. The intermolecular reaction in Miller and Gisvold's experiments yielded only a diazo-amino compound (11), but in the present studies an azo dye also was obtained as a side reaction. The heating period employed by Miller and Gisvold to enhance the yields of the desired cyano compound was eliminated because this step when applied to the preparation of the 2-alkoxy-4-cyano benzoic acids resulted in considerable polymerized material. These compounds were decolorized in ethanol and crystallized from water.

2-Alkoxy-4-cyano Benzoyl Chlorides (VIII).—These compounds were prepared by refluxing the dried acid in an excess of thionyl chloride for one hour. The excess thionyl chloride was removed under vacuum and the resulting acid chloride stored under anhydrous conditions due to the ease of hydrolysis of this type of compound.

Benzyl-2-alkoxy-4-cyano Benzoates (IX).—These esters were prepared by essentially the same method as described by Boblitt and Gisvold (12) in which one equivalent of the acyl chloride and two equivalents of benzyl alcohol were placed in dry benzene and refluxed. The reflux times were 1.25, 5.67, 6.0, and 14 hours respectively for the formation of the benzyl esters of the methoxy, ethoxy, propoxy, and butoxy analogs. The esters were purified by crystallization from ether and petroleum ether and dried under vacuum over phosphorus pentoxide.

Benzyl 2-alkoxy-4-carbethoxyimido Benzoate Hydrochlorides (X).—These imidic esters were prepared according to methods previously described (1, 2).

4-Carbobenzoxy-3-alkoxy Benzamidine Hydrochlorides (XI).—The method of Di Gangi (1) "Method 2" was employed with the following modifications. A large excess of 28% ammonia was added to ethanol before the addition of the imidic ester in accord with the technique used by Boblitt (12). Reflux temperatures were employed in contrast to the lower temperatures of (69–70°) employed by Di Gangi. The amidine hydrochlorides were crystallized from absolute ethanol, acetone, and anhydrous ether. Special precaution was taken during filtration of the purified compounds, because of their hydroscopic nature which was most pronounced in the case of the propoxy and butoxy analogs.

TABLE I.—2-ALKOXY-4-CYANO BENZOIC ACIDS

No.	Ether Group	Yield, %	m. p. °C.	Analysis Calcd.	% C and H Found
1	Methoxy	..	178–180	C, 61.01 H, 3.98	C, 61.06 H, 4.19
2	Ethoxy	59.1	174–176	....	....
3	Propoxy	70.5	131–133	....	....
4	Butoxy	32.5	97–100	....	....

TABLE II.—BENZYL 2-ALKOXY-4-CYANO BENZOATES

No.	Ether Group	Yield, %	m. p. °C.	Analysis Calcd.	% C and H Found
1	Methoxy	75.0	70–72	C, 71.90 H, 4.90	C, 71.78 H, 5.00
2	Ethoxy	66.0	42–45	....	....
3	Propoxy	56.5	30–32	....	....
4	Butoxy	81.4	39–41	....	....

TABLE III.—BENZYL 2-ALKOXY-4-CARBETHOXYIMIDO BENZOATE HYDROCHLORIDES*

No.	Ether Group	Yield, %	m. p. °C.	Analysis Calcd.	% C and H Found
1	Methoxy	80.00	136–137	C, 60.25 $\frac{1}{2}H_2O$ H, 5.90 H_2O	C, 59.93 H, 5.83
2	Ethoxy	43.8	136–137	.....	....
3	Propoxy	73.0	132–133	.....	....
4	Butoxy	33.0	123–125	.....	....

* Sample very resistant to complete drying.

TABLE IV.—4-CARBOBENZOXY-3-ALKOXY BENZAMIDINE HYDROCHLORIDES

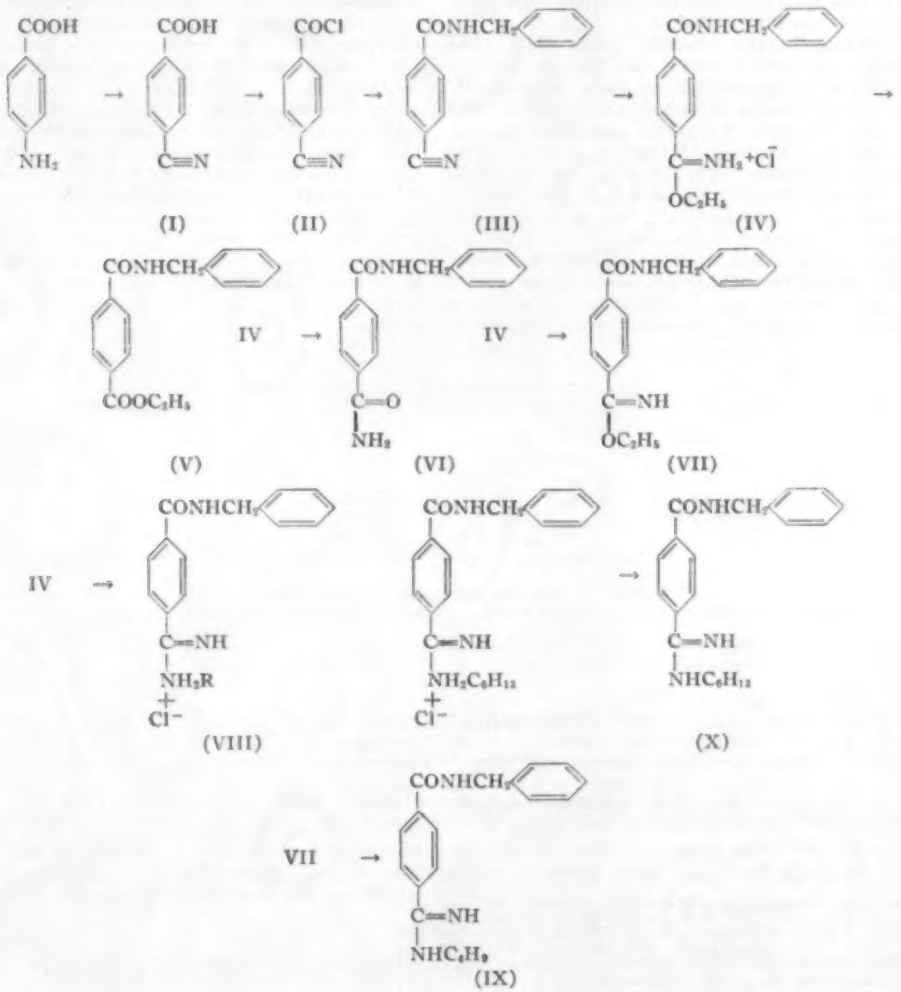
No.	R	R'	Yield, %	m. p. °C.	Analysis Calcd.	% C and H Found
1	Methoxy	H	75.0	196–197	C, 59.90 H, 5.34	C, 59.83 H, 5.72
2	Ethoxy	H	73.7	155–158	C, 60.98 H, 5.72	C, 60.69 H, 5.98
3	Propoxy	H	43.5	99–102	C, 61.97 H, 6.07	C, 61.90 H, 6.42
4	Butoxy	H	60.0	85–89	C, 62.89 H, 6.39	C, 63.00 H, 6.51
5	Ethoxy	n-C_4H_9	32.0	123–125	C, 64.52 H, 6.96	C, 64.18 H, 7.19
6	Propoxy	n-C_4H_9	37.7	136–140	C, 65.25 H, 7.22	C, 65.17 H, 7.72

EXPERIMENTAL (PART II)

The following reactions serve to illustrate the sequence of reactions that are described in this experimental part.

p-**Cyanobenzoic Acid (I).**—The improved method of Miller and Gisvold (11) was utilized for the preparation of this starting compound.

p-**Cyanobenzoyl Chloride (II).**—This acyl chloride was prepared in 96% yield by a method previously described (1, 2).



N-Benzyl *p*-cyanobenzamide (III).—A method essentially the same as that described by Miller (13) "Method B" was used in the preparation of this amide. A total volume of 700 cc. of dry benzene was employed in the modified technique herein used. The reactants used were *p*-cyanobenzoyl chloride (.20 mole) and benzylamine (.43 mole). The mixture was refluxed on a steam pot for three hours and then was allowed to cool in a refrigerator. A solid crystalline product was recovered by filtration, washed thoroughly with water and dried on a Büchner funnel. An additional crop of crystals was recovered from the filtrate. A yield of 41.5 Gm. (87.8%) was thus obtained. The compound was sufficiently pure (m. p. 149–151°) to be used as an intermediate. When a purified sample was desired, recrystallization from ethanol yielded a compound that melted at 151–153°.

N-Benzyl *p*-Carbethoxyimido Benzamide Hydrochloride (IV).—This intermediate was prepared according to the method reported by Chow and Gisvold (2) in which dioxane was used as a solvent. Since an oil was obtained upon addition of petroleum ether, several subsequent washings with petroleum ether and anhydrous ether were necessary to obtain a solid crystalline imidic ester. This resulted in yields of 70% or less.

By using a method similar to the original method of Pinner (14) yields of 93.4% and above were obtained. The method essentially is as follows: 11.5 Gm. of N-benzyl-*p*-cyano-benzamide (m. p. 150–152°) was placed in 150 cc. of absolute alcohol and 50 cc. of anhydrous ether. The solution was cooled to 0° to 5° and treated with dry hydrogen chloride gas for three hours. The solution was then placed in a refrigerator protected from moisture for five days. An excess of dry ether was then added aud the mixture was returned to the refrigerator for two hours. The crystalline solid which separated was recovered by filtration on a Büchner funnel and washed with dry ether (m. p. 245–250°).

When desired the imidic ester hydrochloride was

TABLE V.—*p*-SUBSTITUTED N-BENZYL BENZAMIDE X-$C_6H_4CONHCH_2C_6H_5$

No.	X	Yield, %	m. p. °C.	Analysis Calcd.	% C and H Found
1	Cyano	87.8	150–151	C, 76.25 H, 5.12	C, 75.83 H, 5.41
2	Carbethoxyimido HCl	93.4	250–251	C, 64.04 H, 6.01	C, 64.15 H, 6.15
3	Carbethoxyimido	..	126–128	....	....
4	Amido	..	250–252	C, 71.12 H, 5.55	C, 71.01 H, 5.64
5	Carbethoxy	..	128–129	C, 72.06 H, 6.05	C, 72.59 H, 6.10

TABLE VI.—N-BENZYL *p*-N′-*n*-ALKYLAMIDINO BENZAMIDE HYDROCHLORIDES HCl.NHR-$C(NH)C_6H_4CONHCH_2C_6H_5$

No.	R	Yield, %	m. p. °C.	Analysis Calcd.	% C and H and N Found
1	H[a]	..	130–180	C, 57.68 H, 5.97 N, 13.45	C, 57.87 H, 6.47 N, 13.91
2	N-propyl	82.8	225–228	C, 65.15 H, 6.68	C, 65.37 H, 6.91
3	*n*-butyl	82.6	257–259	C, 65.97 H, 6.99 N, 12.15	C, 65.60 H, 7.39 N, 11.98
4	*n*-amyl	88.2	189–191	C, 66.72 H, 7.28	C, 66.98 H, 7.14
5	*n*-hexyl	71.0	166–168	C, 67.45 H, 7.55	C, 67.38 H, 7.68
	N-Benzyl *p*-N′-*n*-Hexylamidino Benzamide Base				
1	*n*-hexyl	..	132–134	C, 74.74 H, 8.36 N, 12.45	C, 74.44 H, 8.32 N, 12.35

[a] Prepared according to the method of Di Gangi (1) using ethanol as a reaction medium.

TABLE VII.—*p*-SUBSTITUTED BENZYL THIOBENZOATE X-C_6H_4-$COSCH_2C_6H_5$

No.	X	Yield, %	m. p. °C.	Analysis Calcd.	% C and H or N Found
1	Cyano	75.4	69–72	C, 71.12 H, 4.38	C, 70.91 H, 4.50
2	Carbethoxyimido HCl	93.1	173–176	....	....
3	Carbethoxy		55	C, 67.98 H, 5.37	C, 68.17 H, 5.62
4	Carbethoxyimido base		56–58	N, 4.68	N, 4.49
5	Amido		175–177	N, 5.61	N, 4.87
6	*n*-Butylamidino HCl		170–179	C, 62.88 H, 6.39	C, 62.83 H, 6.62

purified by dissolving it in cold absolute ethanol and anhydrous ether then added to induce crystallization. In this manner a product that melted at 250–252° was obtained.

N-Benzyl *p*-Carbethoxy Benzamide (V).—When a purified sample of N-benzyl *p*-carbethoxyimido benzamide hydrochloride was placed in water the compound dissolved instantly. Within ten seconds platelets of the corresponding ester began to appear. Within ten minutes a flocculent precipitate had filled the container. It was removed by filtration, dried, and crystallized from ether and petroleum ether.

N-Benzyl *p*-Amido Benzamide (VI).—When a sample of N-benzyl *p*-carbethoxyimido benzamide hydrochloride was refluxed in either absolute ethanol or dry dioxane for several hours, N-benzyl *p*-amido benzamide was recovered. Purification was carried out by crystallization from absolute ethanol.

N-Benzyl *p*-Carbethoxyimido Benzamide Base (VII).—This compound was prepared from the corresponding hydrochloride by the method of Glickman and Cape (15).

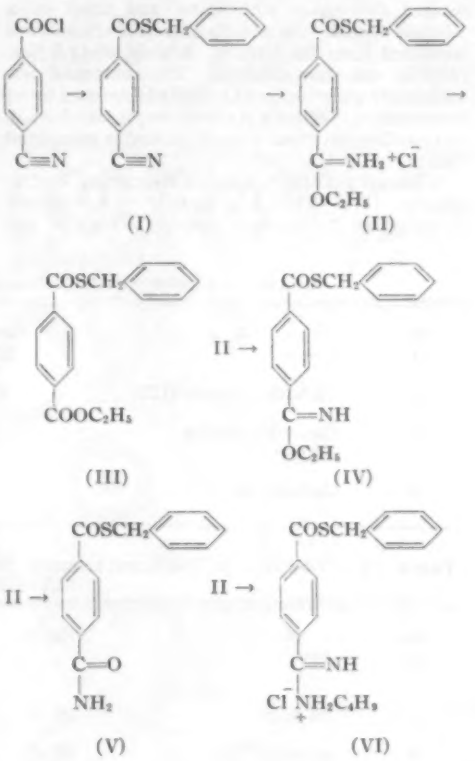
N-Benzyl *p*-N′-*n*-Alkylamidino Benzamide Hydrochlorides (VIII).—Since previous methods of Pinner (1, 14) gave low yields and presented difficulty in purification, a new method of preparation of alkyl substituted amidines was developed. An outline of this method is as follows: 0.01 mole of N-benzyl *p*-carbethoxyimido benzamide hydrochloride was placed in 200 cc. of *p*-dioxane (Eastman Kodak) containing 0.02 mole of the appropriate amine. The mixture was refluxed on a steam pot for two to three hours, cooled and the crystals were removed by filtration. The compound was usually quite pure at this point. If further purification was desired, the compound was crystallized from absolute ethanol, acetone, and anhydrous ether. In the preparation of N-benzyl *p*-N′-*n*-benzylamidino benzamide hydrochloride, an oil was obtained at the end of the reflux period. This oil solidified after being air dried for one week. Crystallization from absolute ethanol, acetone, and anhydrous ether gave a light fluffy crystalline compound.

N-Benzyl *p*-N′-*n*-Butylamidino Benzamide Base (IX).—N-benzyl *p*-carbethoxyimido benzamide base was prepared as previously outlined (15). This compound was added to *p*-dioxane containing two equivalents of butylamine and refluxed for three hours. Dry hydrogen chloride dissolved in *p*-dioxane was then added. The crystalline compound which appeared was recovered and identified as the starting imidic ester. The reaction was unsuccessful indicating that the presence of acid is necessary for the formation of this amidine from the corresponding imidic ester and the appropriate alkyl amine.

N-Benzyl *p*-N′-*n*-hexylamidino Benzamide Base (X).—According to the method of Di Gangi and Gisvold (1) a 10% solution of sodium hydroxide was employed to obtain the amidine base from the hydrochloride. The base was then extracted with chloroform, etc. The following sample is offered as an alternate method for obtaining the amidine base in good yield and in a pure state: A small sample of N-benzyl *p*-N′-*n*-hexylamidino benzamide hydrochloride was dissolved in water. The corresponding amine, hexylamine in this case, was added dropwise with shaking. The basic amidine which precipitated was recovered by filtration, washed thoroughly with water and dried.

EXPERIMENTAL (PART III)

The following reactions serve to illustrate the sequence of reactions that are described in this experimental part.



The imidic ester hydrochloride was purified by first converting it to the imidic ester base (15) from which the imidic ester hydrochloride was obtained by the addition of dry hydrogen chloride in dioxane, to a solution of the imidic ester base in dioxane.

Benzyl *p*-Cyano Thiobenzoate (I).—This intermediate was synthesized from *p*-cyano benzoyl chloride and an excess of benzyl mercaptan by heating the reactants on a steam pot until hydrogen chloride fumes were no longer evolved. An excess of ice chips and water was then added to the mixture and the solid crystals were recovered by filtration. The solid was crystallized from absolute ethanol and then from petroleum ether yielding beautiful snowflake-like crystals.

Benzyl *p*-Carbethoxyimido Thiobenzoate Hydrochloride (II).—The method previously reported (1, 2) was applied in the preparation of this compound.

Benzyl *p*-Carbethoxy Thiobenzoate (III).—A small sample of benzyl *p*-carbethoxyimido thiobenzoate hydrochloride was placed in water and brought to boiling. The mixture was quickly filtered and the filtrate placed in a refrigerator. The

crystals were recovered and recrystallized from ether and petroleum ether.

Benzyl *p*-Carbethoxyimido Thiobenzoate Base (IV).—The method of Glickmann (15) was utilized in preparing this compound from the corresponding imidic ester hydrochloride. The base was also obtained by refluxing the imidic ester hydrochloride in dry dioxane and an excess of dry ammonia.

Benzyl *p*-Amido Thiobenzoate (V).—By refluxing the imidic ester hydrochloride in absolute ethanol for three hours, the corresponding amide was obtained. The compound was crystallized from absolute ethanol.

Benzyl *p*-N-*n*-Butylamidino Thiobenzoate Hydrochloride (VI).—The usual methods of preparation (1, 14) failed to yield the desired amidine. The dioxane method introduced in this paper yielded the desired compound.

SUMMARY

1. Six new 4-carbobenzoxy-3-alkoxy benzamidine hydrochlorides were prepared, two of which contained an *n*-butyl substituent on the amidino group.

2. Four new 2-alkoxy-4-cyano benzoic acids and their corresponding benzyl esters were prepared.

3. Four new benzyl 2-alkoxy-4-carbethoxyimido benzoate hydrochlorides were synthesized.

4. Five new N-benzyl *p*-N′-*n*-alkylamidino benzamide hydrochlorides were prepared. Of these compounds the N-benzyl *p*-N′-*n*-hexylamidino benzamide base was prepared.

5 Five new *p*-substituted benzyl benzamides were prepared.

6. Benzyl *p*-N-*n*-butylamidino thiobenzoate hydrochloride was prepared.

7. Five new *p*-substituted benzyl thiobenzoates were prepared.

8. A new and improved simplified method for the preparation of N-benzyl-*p*-N′-*n*-alkylamidino benzamide hydrochlorides has been devised. The formation *of ortho* esters and esters is avoided by the substitution of dry dioxane for ethanol when the substituted amidine is prepared from the amino ether hydrochloride. In addition amide formation is minimized by the addition of the base to the imino ether hydrochloride before the mixture is heated.

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Isonicotinyl Hydrazones from D-Mannuronolactone and D-Mannuronic Acid*

By CRAWFORD H. BROWN, H. EDWARD BOND, S. ANDERSON PEOPLES, and PETER P. T. SAH†

D-Mannuronolactone isonicotinyl hydrazone and D-mannuronic acid isonicotinyl hydrazone, two new compounds highly active against *Mycobacterium tuberculosis* H37Rv, have been prepared from alginic acid as the starting material.

THE SYNTHESIS of D-glucuronolactone isonicotinyl hydrazone with an excellent yield by the condensation of D-glucuronolactone with isonicotinic acid hydrazide in absolute methanol as the solvent was reported by one of us (1). The antituberculosis activity of this new drug in animals and in humans was studied by Cabot Brown, *et al.*, (2), who reported that it is more effective than streptomycin and less toxic than isonicotinic acid hydrazide. These chemical and clinical results have been confirmed completely and independently by other groups of international research workers (3–6).

The synthesis of D-galacturonic acid isonicotinyl hydrazone with an equally excellent yield by the condensation of D-galacturonic acid with isonicotinic acid hydrazide in distilled water or dilute methanol as the solvent was also reported by us in THIS JOURNAL (7). Biological

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studies revealed that this new agent is even more effective and at the same time less toxic than its analog, D-glucuronolactone isonicotinyl hydrazone.

D-Mannuronolactone may be obtained by the hydrolysis of alginic acid (8). The hydration of D-mannuronolactone at high temperature gives rise to D-mannuronic acid which may also be prepared from D-mannosaccharic acid by reduction with sodium amalgam (9, 10). Synthesis of the isonicotinyl hydrazones of these two sugar acid derivatives has not been described in the literature.

This paper deals with (*a*) the preparation of D-mannuronolactone from alginic acid, (*b*) the condensation of the sugar lactone with isonicotinic acid hydrazide to form 1) D-mannuronolactone isonicotinyl hydrazone in absolute alcohol (ethyl or methyl) as the solvent and 2) D-mannuronic acid isonicotinyl hydrazone in distilled water as the solvent, (*c*) the physical properties and chemical analyses of these two new drugs, (*d*) their acute toxicity in comparison with other analogous compounds, and (*e*) their antituberculosis activity.

Detailed information on the pharmacology, toxicology, and experimental therapeutics will be published elsewhere.

EXPERIMENTAL

Preparation of D-Mannuronolactone from Kelacid®[1] or Alginic Acid.—Several different methods of hydrolyzing alginic acid to D-mannuronolactone were tried out in our laboratory. The only method found to be practical was that described by Spoehr (8). Alginic acid powder (Kelacid®, 100 Gm.) was hydrolyzed with boiling 90% formic acid (1600 cc.) under reflux for twenty hours in the hood.

After removal of the hydrolytic agent by vacuum distillation, the crude product was decolorized and recrystallized from a minimum amount of warm water. After filtration and washing with absolute ethyl alcohol and acetone, the purified product weighed 10 Gm. and formed white prisms melting with decomposition between 182 and 185°. The melting points recorded in the literature are: 145° corr. (8), 194° (11), 142–143° (12), and 139° (14).

The specific optical rotation of our product was determined forty minutes after solution at a concentration of 4.00 Gm. per 100 cc. and found to be: $[\alpha]_D^{25}$ 91.5°, which is identical with that obtained from a sample furnished by Kelco Company. The values from the literature are: $[\alpha]_D^{18}$ 92.13° (8), $[\alpha]_D^{20}$ 89° (11).

Anal.—Calcd. for $C_6H_8O_6$: C, 40.9; H, 4.5. Found: C, 40.75; H, 4.77.

Preparation of D-Mannuronic Acid Isonicotinyl Hydrazone.—D-Mannuronolactone (3.52 Gm. or 20 millimoles) was dissolved in hot distilled water (15 cc.). To the hot solution, isonicotinic acid hydrazide (2.74 Gm. or 20 millimoles) dissolved in the same hot solvent (20 cc.) was added. The reaction mixture, after thorough shaking, was allowed to stand at 50° for ten minutes and then left in the refrigerator overnight. The white, crystalline product was then filtered off with suction, washed first with a small amount of ice-cold distilled water and then with 95% ethyl alcohol and finally sucked practically to dryness. After drying in the vacuum desiccator for three days, it weighed 5.4 Gm. (91% of the theoretical yield). The product was easily recrystallized from a minimum amount of boiling distilled water from which it formed white platelets, melting with decomposition between 139 and 140° (uncorr.); 142–143° (corr.).

Anal.—Calcd. for $C_{12}H_{13}O_7N_3$: C, 46.00; H, 4.83; N, 13.42. Found: C, 45.92; H, 4.87; N, 13.40; C, 46.08; H, 4.73; N, 13.56.

Preparation of D-Mannuronolactone Isonicotinyl Hydrazone.—The procedure was essentially the same as given above, except that absolute alcohol (methyl or ethyl) was used as the solvent to avoid the hydration of the product. To dissolve completely 3.52 Gm. of D-mannuronolactone at the boiling point of the solvent, it was necessary to use 550 cc. of absolute ethyl alcohol or 250 cc. of absolute methyl alcohol. To bring the molecular equivalent amount of isonicotinic acid hydrazide to complete solution at 60°, 100 cc. of the solvent (either ethyl or methyl alcohol) was sufficient. The yield of the final product with absolute ethyl alcohol as the solvent was 91%, slightly higher than when absolute methyl alcohol was used.

Pure D-mannuronolactone isonicotinyl hydrazone, after recrystallization once or twice from absolute ethyl alcohol, formed white, fine needles, melting with decomposition between 181 and 182° (uncorr.); 185–186° (corr.).

Anal.—Calcd for $C_{12}H_{13}O_6N_3$: C, 48.79; H, 4.45; N, 14.24. Found: C, 48.57; H, 4.70; N, 14.28.

When the product was recrystallized from hot water or from dilute ethyl alcohol, it took up one mole of water and formed D-mannuronic acid isonicotinyl hydrazone, melting with decomposition between 139 and 140° (uncorr.); 142–143° (corr.).

Acute Toxicity.—The results of our toxicity studies in mice with isonicotinyl hydrazones from D-mannuronolactone and D-mannuronic acid are summarized in Table I which also provides a comparison with the acute toxicities of isonicotinyl hydrazones from D-glucuronolactone and D-galacturonic acid as well as the reagent, isonicotinic acid hydrazide. From the table, it is apparent that the two new chemotherapeutic agents (isonicotinyl hydrazones from D-mannuronolactone and D-mannuronic acid) are like their analogs (isonicotinyl hydrazones from D-glucuronolactone and D-galacturonic acid), much less toxic than the mother drug, isonicotinic acid hydrazide.

Antituberculosis Activity in vivo.—Preliminary biological tests indicate that both D-mannuronolactone isonicotinyl hydrazone and D-mannuronic acid isonicotinyl hydrazone are very close in activity *in vivo* to D-glucuronolactone isonicotinyl hydrazone and D-galacturonic acid isonicotinyl hydrazone against *Mycobacterium tuberculosis* H37Rv. See Table II and compare with our work previously

[1] We wish to thank Kelco Company, Los Angeles 14, Calif., for supplying us the raw material, Kelacid® and samples of D-mannuronolactone for the research.

TABLE I.—ACUTE TOXICITY OF ISONICOTINYL HYDRAZONES DERIVED FROM URONIC ACIDS AND URONOLACTONES

Name of Drug INH = Isonicotinyl Hydrazone	Mean Lethal Dose (LD_{50}) Mice			
	Oral		I. P.	
	mg./Kg.	mM./Kg.	mg./Kg.	mM./Kg.
D-Mannuronolactone INH	1100 ± 100	3.7	1400 ± 150	4.7
D-Mannuronic acid INH	1000 ± 250	3.2	1500 ± 250	4.8
D-Glucuronolactone INH	1100 ± 100	3.7	2000 ± 200	6.8
D-Galacturonic acid INH	1400 ± 200	4.5	2200 ± 100	7.0
Isonicotinic acid hydrazide (INAH or Isoniazid)[a]	160 ± 40	1.2	145 ± 30	1.1

[a] The LD_{50} of isonicotinic acid hydrazide, mg./Kg. mice oral, was reported by Benson, *et al.* (14), Rubin, *et al.* (15), and P'an, *et al.* (16), to be 142 ± 10, 100 ± 6, and 170 ± 5 respectively. The LD_{50} of the same drug, mg./Kg. mice I. P., was reported by Benson, *et al.* (14) to be 132 ± 13.2. Our toxicity studies covering a period of more than two years and involving the use of several hundred animals and the close team work of several laboratories agree very closely with the findings reported in the literature (17).

TABLE II.—IN VIVO ANTITUBERCULOSIS ACTIVITY OF ISONICOTINYL HYDRAZONES DERIVED FROM URONIC ACIDS AND URONOLACTONES IN MICE INFECTED WITH MYCOBACTERIUM TUBERCULOSIS H37Rv

Compounds Tested	Dosage in Diet, %	Number of Mice	Days on Test	Mortality, %	Mean Survival Time in Days
D-Mannuronolactone INH	0.1	5	28	0	28+
	0.05	5	28	0	28+
	0.025	5	28	20	27−
D-Mannuronic acid INH	0.1	5	28	0	28+
	0.05	5	28	0	28+
	0.025	10	28	10	27+
D-Glucuronolactone INH	0.1	5	28	0	28+
	0.05	10	28	0	28+
	0.025	10	28	10	27+
D-Galacturonic acid INH	0.1	5	28	0	28+
	0.05	10	28	0	28+
	0.025	10	28	0	28+
Isonicotinic acid hydrazide (INAH or Isoniazid) (standard for comparison)	0.2[a]	10	28	50	20−[b]
	0.1	10	28	10	27[b]
	0.05	10	28	0	28+
	0.025	10	28	20	26−
Streptomycin (standard)	3000 units weekly by s.c. inj.	10	28	20	27−
Control	No drug	10	28	90	16−

[a] The daily intake of the drug by the mouse weighing about 15 Gm. was estimated to be about 120 mg./Kg.
[b] Animals died of drug toxicity (symptoms of convulsions) rather than of tuberculosis. The test was carried out according to standard procedures described in the literature (read references given in the paper previously published by Sah and Peoples (7) in THIS JOURNAL).

published (1, 7). Research is still in progress. Detailed information will be available for publication when the investigations are complete.

SUMMARY

1. Procedures for the preparation of D-mannuronolactone isonicotinyl hydrazone and D-mannuronic acid isonicotinyl hydrazone from alginic acid are described.

2. These two new chemotherapeutic agents against tuberculosis are found to possess chemotherapeutic properties similar to D-glucuronolactone isonicotinyl hydrazone and D-galacturonic acid isonicotinyl hydrazone. They are more effective than streptomycin and less toxic than isonicotinic acid hydrazide in the treatment of mice infected with *Mycobacterium tuberculosis* H37Rv.

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The Influence of Thiamine on the Microbiological Assay for Vitamin B_{12} with *Escherichia coli* 113–3*

By J. M. McLAUGHLAN, C. G. ROGERS, and J. A. CAMPBELL

The tube assay employing *Escherichia coli* 113–3 with the medium of Davis and Mingioli gave low estimates of potency for vitamin B_{12} in aqueous extracts of multivitamin preparations when compared with the U. S. P. method. The presence of thiamine in the multivitamin samples was found to be responsible for these low values which were most evident in the shaken assays. When samples were extracted with sodium *meta*-bisulfite to destroy the thiamine, the potency estimates agreed well with those obtained with the U. S. P. procedure. This extraction eliminated the need for cyanide treatment. In view of its stability of response and greater simplicity as compared with the U. S. P. assay, the *E. coli* method seems particularly applicable to the estimation of vitamin B_{12} activity in the absence of interfering materials, *e. g.*, pseudo-forms of vitamin B_{12} or methionine.

MANY different microbiological procedures for the estimation of vitamin B_{12} have been proposed during the seven years since its isolation. Of these methods, one employing *Lactobacillus leichmannii* (A.T.C.C. 7830) has been most widely adopted. It is the official method in the United States (1, 2) and is also used in Canada for the assay of vitamin B_{12} in pharmaceuticals. Along with other workers, the authors (3) have found that it yields precise and reproducible results but suffers from the disadvantage that the organism is extremely sensitive to the way it is grown and prepared for assay. It has also been shown (4, 5) that glassware, for use in this assay, must be cleaned with considerable care.

Several recent publications (6, 7, 8, 9) have shown the advantages of *Escherichia coli* 113–3 as an assay organism for vitamin B_{12} in samples with a low methionine content. The *E. coli* methods are relatively simple to handle and the media are inexpensive. Chiao and Peterson (7), using the medium of Davis and Mingioli (10), showed that the response of *E. coli* to vitamin B_{12} was remarkably stable from one assay to another. They recommended a shaken assay, whereas Johansson (8) found that the stationary assay was more convenient and precise. In this laboratory preliminary comparisons of aqueous extracts of multivitamin pharmaceuticals indicated that the *E. coli* stationary assay yielded lower results than the *L. leichmannii* method. This was difficult to understand since many products contained crystalline vitamin B_{12} which was the same as the reference standard. Further studies suggested that thiamine lowered the response of *E. coli* to vitamin B_{12}. It was decided, therefore, to investigate the effect of thiamine and other B-vitamins commonly present in vitamin mixtures, on the response of *E. coli* to vitamin B_{12}, and to attempt to modify this procedure for the assay of multivitamin products.

* Received April 16, 1955, from the Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Canada.

Presented, in part, before The Annual Convention of The Chemical Institute of Canada, June 21–23, 1954, Toronto, Canada.

GENERAL PROCEDURE

The *E. coli* method used was essentially that of Chiao and Peterson (7). The *L. leichmannii* method was the U. S. P. procedure (1) modified by the use of the 6 point design (3). Samples were extracted with a buffered 1% solution of sodium *meta*-bisulfite (2). The cyanide treatment was that described by Chiao and Peterson. For purposes of comparison, both *L. leichmannii* and *E. coli* assays were run simultaneously. After extraction of the samples, the same solutions were used in both procedures. Final dilutions for the *E. coli* assay were diluted 1:5 for the *L. leichmannii* assay. In both methods tubes were randomized throughout the racks just before addition of the basal medium.

In the *E. coli* assay glucose was sterilized with the basal medium and tubes (20 x 150 mm.) were autoclaved at 120° for three to four minutes. Preliminary investigations showed, that by the use of a shorter sterilization time it was possible to autoclave the glucose in the assay medium without appreciably lowering the growth response to vitamin B_{12}. For the modified medium, thiamine was added at the rate of 0.2 μg. per tube. In most tests 18 tubes were employed on the reference standard and from 9–12 tubes on each sample or test preparation. Shaken assays were run on a Yankee Kahn Test shaker at a speed of 275 oscillations per minute. Both shaken and stationary assay cultures were incubated for sixteen hours at 31°. Turbidities were read on the Coleman Spectrophotometer model 11 at 540 mμ and readings were converted to the absorbancy values. The response curves were plotted on an arithmetic scale.

EXPERIMENTAL RESULTS

Effects of B-Vitamins.—Table I shows the effect of several of the B-vitamins on the response of the *E. coli* mutant in shaken and stationary assays.

Six B-vitamins were tested at three levels in each type of assay and the proportions of these vitamins to vitamin B_{12} included those usually found in pharmaceuticals. Higher concentrations were used in the shaken assay since the dilution of vitamin B_{12} was less. In each test a solution of vitamin B_{12} containing the vitamin under test was assayed against the same solution of vitamin B_{12} alone. It is evident that thiamine was the only vitamin which had any effect on the response of the *E. coli* mutant to vitamin B_{12}; this effect was most evident in the shaken assay. The growth depressing effect of thiamine was not large in stationary assays, but repeated tests confirmed the fact that the effect was real, and amounted to about 5 per cent.

The full growth response curves for the test organism in shaken assays with and without thiamine are shown in Fig. 1 along with a typical growth response curve for the stationary assay without added thiamine. It is apparent that thiamine, in the shaken assay, lowered the response of the *E. coli* mutant to vitamin B_{12} by approximately 25 per cent. The response to vitamin B_{12} is linear over the range of 0.1–0.6 mμ per tube in the shaken assay and from 0.1–0.3 mμ in the stationary assay.

Since the *E. coli* basal medium was buffered at *p*H 6.8 it seemed possible that the effect of thiamine may have resulted from partial destruction of vitamin B_{12} (11). However, from an investigation of the stability of vitamin B_{12} in the modified *E. coli* basal medium at the incubation temperature, 31°, it was found that vitamin B_{12} was reasonably stable in the presence of thiamine under conditions similar to those of the vitamin B_{12} assay. The drop in vitamin B_{12} potency was only about 10 per cent after twenty-one days storage.

The difference in growth depression between shaken and stationary assays was also used to study the effect of thiamine. If the apparent depressing effect of thiamine resulted from destruction of vitamin B_{12} then, presumably, the destruction would be accelerated by aeration. Therefore, the effect should have appeared if tubes were shaken for sixteen hours prior to inoculation and incubation as a stationary assay. However, results of several tests indicated that the depression of response was characteristic of the type of assay, i. e., shaken or stationary, and was not influenced by the treatment prior to incubation. These tests confirmed the conclusion that thiamine does not destroy vitamin B_{12} under the conditions of the assay, whether tubes are shaken or stationary.

Concentration of Thiamine.—It was noted that the three levels of thiamine (Table I) exhibited the same growth depression, hence, it was of interest to determine the degree of depression of response over a wider range of thiamine concentrations. Figure 2 shows the effect of various levels of thiamine on the apparent potency of vitamin B_{12}. Each point consisted of an assay of a solution of vitamin B_{12} with the indicated amount of thiamine, against a standard of vitamin B_{12} alone. The test was made only with shaken assays in which the effect of thiamine was greatest. It appeared that concentrations of thiamine above 0.1 μg. per tube had no additional effect. This suggested that, if thiamine were added to the basal medium at the rate of 0.1 μg. per tube, the thiamine originally present in multivitamin preparations would have no further effect and a correct estimate of vitamin B_{12} potency should be obtained.

Extraction with Sodium Meta-Bisulfite.—Both the A. O. A. C. (2) and the U. S. P. (1) recommend extraction of samples with a buffered 1% solution of

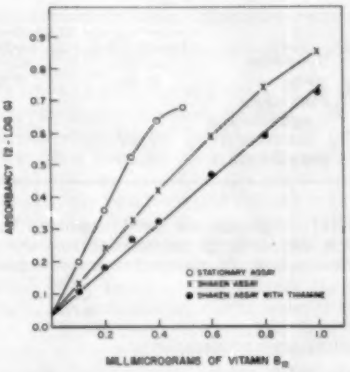

Fig. 1.—Response of *E. coli* 113-3 to vitamin B_{12}.

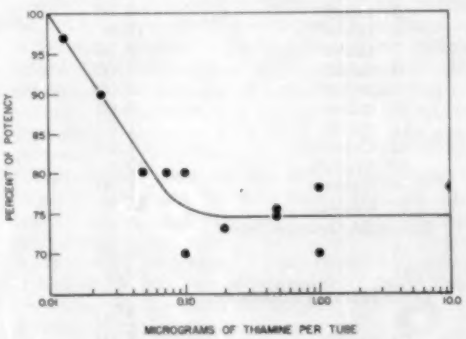

Fig. 2.—Effect of thiamine on apparent potency of vitamin B_{12} standard in shaken assays.

TABLE I.—THE EFFECT OF SINGLE VITAMINS ON THE E. COLI 113-3 RESPONSE TO VITAMIN B_{12}

	Per Cent Recovery Of Vitamin B_{12}					
	Stationary Assay			Shaken Assay		
Vitamin μg/tube	0.05	0.25	0.50	0.10	0.50	1.00
Thiamine	95	95	97	77	74	76
Riboflavin	102	101	100	103	99	102
Niacinamide	100	98	98	102	105	101
Pyridoxine	99	100	98	98	98	98
Ca *d*-Pantothenate	98	98	98	98	99	99
Folic acid	100	101	100	100	101	93

sodium *meta*-bisulfite prior to assay with *L. leichmannii*. For this reason, a study was made to determine if this extraction procedure might also be applied to samples assayed with the *E. coli* mutant. It was found that sodium *meta*-bisulfite, at the concentrations per tube that would normally be used in the assay of most pharmaceuticals, where the dilution factor is relatively large, did not effect the response of *E. coli* to vitamin B_{12}.

Chiao and Peterson (7) reported that vitamin B_{12b} had about 75 per cent of the activity of vitamin B_{12} when assayed by the *E. coli* method, but after cyanide treatment vitamin B_{12b} was fully as active as vitamin B_{12}. These workers also reported that it was necessary to remove excess cyanide because of the sensitivity of the *E. coli* mutant to this reagent. In the present study an investigation was made to determine the effect of *meta*-bisulfite extraction on the potency of vitamin B_{12b} as estimated with the *E. coli* mutant. The results, presented in Table II, show that the potency of vitamin B_{12b} was the same after *meta*-bisulfite extraction or cyanide treatment and agreed well with the estimate of potency by the *L. leichmannii* assay.

TABLE II.—THE EFFECT OF DIFFERENT TREATMENTS ON THE APPARENT POTENCY OF VITAMIN B_{12b}

Sample	Treatment	Assay Method—*E. coli* 113–3, µg./ml.	Assay Method—*L. leichmannii*, µg./ml.
B_{12b}	None	0.69	1.01
B_{12b}	Potassium cyanide and aeration	1.07	1.07
B_{12b}	*meta*-Bisulfite	1.10	1.09

Comparison of Methods:—The reliability of the *E. coli* 113–3 stationary assay for vitamin B_{12} was assessed by comparison with the U. S. P. method using *L. leichmannii*. Samples, prior to assay, were extracted with a buffered solution of sodium *meta*-bisulfite. Assays by the two methods were run simultaneously. In the first series of assays, the medium of Davis and Mingioli (10) was modified by the addition of thiamine; in the second series thiamine was omitted. The results of the first set of assays are presented in Table III, and of the second set, in Table IV. Most of the estimates of potency reported were obtained from single analyses. The deviation, in per cent, of the *E. coli* estimate from that by the U. S. P. *L. leichmannii* method is shown for each product.

The results presented in Table III show that for almost all products, potency estimates obtained by

TABLE III.—COMPARISON OF METHODS. VITAMIN B_{12} POTENCY OF PHARMACEUTICALS ASSAYED AFTER SODIUM META-BISULFITE EXTRACTION AND ADDITION OF THIAMINE TO THE E. COLI ASSAY MEDIUM.

	Label Claim µg./tab., cap. or cc.	Method of Assay—*E. coli* 113–3, µg.	Method of Assay—*L. leichmannii*, µg.	Deviation from *L. leichmannii*, %
Multivitamin preparations				
1 elixir	0.35	0.29	0.28	+3.6
2 elixir	0.20	0.11	0.12	−8.3
3 elixir	1.25	1.59	1.52	+4.6
4 elixir	0.60	0.53	0.55	−3.6
5 tablet	6.00	5.00	4.40	+13.6
6 tablet	10.0	5.40	5.50	−1.8
7 tablet	0.50	0.45	0.43	+4.7
8 tablet	2.00	1.60	1.60	0.0
9 tablet	5.00	4.00	4.00	0.0
10 tablet	5.00	2.43	2.32	+4.7
11 tablet	1.67	1.19	1.25	−4.8
12 Capsule	1.00	0.85	0.84	+1.2
13[a] Capsule	10.0	10.7	10.8	−0.9
14[a] Capsule	1.00	0.92	0.82	+12.2
15[a] Capsule	15.0	13.6	14.3	−4.9
Injectable concentrates				
16	30.0	21.8	22.0	−0.9
17	30.0	26.0	25.5	+2.0
Liver extracts injectable				
18	2.00	2.73	3.20	−14.7
19	2.00	2.60	2.40	+8.3
20	10.0	22.0	23.2	−5.2
21	20.0	36.0	34.3	+5.0
22	10.0	31.5	31.0	+1.6
23	20.0	34.0	30.5	+11.5
24	20.0	26.0	25.0	+4.0
25	20.0	26.0	25.5	+2.0
26	20.0	23.0	24.4	−5.7
Natural materials[b]				
27 (B_{12} and pseudo B_{12} mix)		0.775	0.952	−18.6
28 (V_{12} with Intrinsic Factor Concentrate)		424.0	417.0	+1.7
29 (Crude B_{12} fermentation product)		113.0	18.7	+504.0
30 (Commercial grade condensed fish solubles)		0.229	0.225	+1.8

[a] Contained Intrinsic Factor Concentrate.
[b] Samples Assayed in 1954 A. O. A. C. Vitamin B_{12} Collaborative Study.

TABLE IV.—COMPARISON OF METHODS. VITAMIN B_{12} POTENCY OF PHARMACEUTICALS AFTER SODIUM META-BISULFITE EXTRACTION.

	Label Claim, μg./tab., cap. or cc.	Method of Assay		Deviation from *L. leichmannii*, %
		E. coli 113-3, μg.	*L. leichmannii*, μg.	
Multivitamin preparation				
1	1.25	1.21	1.25	−3.2
2	0.60	0.18	0.19	−5.3
3[a]	4.00	3.50	4.40	−20.5
		(0.25)[b]	(0.62)[b]	
4	1.75	2.10	2.00	+5.0
Known mixtures[c]				
A	1.00	1.00	—	—
B	1.00	0.97	—	—
C	1.00	0.99	—	—

[a] Contained dried yeast.
[b] Residual activity after alkali treatment.
[c] Contained varying proportions of B-vitamins and ascorbic acid.

the two methods were in good agreement. As was expected, a large difference in response was obtained with sample 29, a crude vitamin B_{12} fermentation product. With the possible exception of samples 5, 14, 18, and 29, the deviation of the modified *E. coli* method from the U. S. P. procedure fell well within the limits of experimental error for these microbiological assays.

It was evident from Table IV that the *E. coli* assay gave equally reliable results when thiamine was omitted from the Davis and Mingioli medium. Chemical analyses of standard solutions of thiamine by the thiochrome method before and after extraction had shown that thiamine was completely destroyed by *meta*-bisulfite. The higher value for the vitamin B_{12} potency of sample 3 obtained by the *L. leichmannii* method, was probably caused by the presence of deoxyribosides in the dried yeast as evidenced by the residual activity shown by this product after alkali treatment (13).

The fact that many of the pharmaceutical preparations used in this study were found to be below label claim substantiates other data from this laboratory (14, 17) indicating a need for closer control of the potency of these products.

DISCUSSION

Several workers (7, 9) have outlined the possible advantages of *E. coli* 113-3 as an assay organism for vitamin B_{12} in pharmaceuticals. However, few multivitamin preparations appear to have been tested. The present investigation indicated that thiamine lowered the growth response of *E. coli* to vitamin B_{12}, and estimates of potency for vitamin B_{12} in aqueous extracts of multivitamin products were lower by the *E. coli* assay than by the *L. leichmannii* method particularly in shaken assays. Although the effect of thiamine has been the subject of some study, the reason for its growth depressing action is not known. Destruction of Vitamin B_{12} does not seem to be involved. Dubnoff (18) has suggested that vitamin B_{12} maintains R–SH compounds in the reduced state. Taizo Matsukawa and Shojiro Yurugi (19) have indicated that the disulfide of thiamine oxidizes R–SH compounds such as glutathione. Evidence obtained in our laboratory suggests that the disulfide of thiamine is formed under highly aerobic conditions, and this results in a retardation of the growth rate when vitamin B_{12} is limiting.

The effect of thiamine was not evident when the medium of Burkholder was employed. A similar effect was observed by Hoff-Jorgensen (15) who reported that proteins and certain amino acids depressed the response of *E. coli* 113-3 to vitamin B_{12} but that the effect was not evident in the Burkholder medium. Since the medium of Davis and Mingioli is simpler than the Burkholder medium the former was used in this investigation. Although the effect of thiamine on the vitamin B_{12} potency estimates was overcome by the addition of thiamine to the assay medium, this resulted in a lowered sensitivity of the test organism to vitamin B_{12}.

The use of sodium *meta*-bisulfite for the extraction of samples was found preferable to the addition of thiamine, since it destroyed thiamine present in samples and did not affect the response to vitamin B_{12}. The action of sodium *meta*-bisulfite on thiamine is probably identical with that caused by sodium sulfite (12). Extraction with *meta*-bisulfite also eliminated the need for the cyanide treatment of samples; furthermore it is the accepted method of extraction in both the A. O. A. C. and U. S. P. procedures.

The present findings are in agreement with those of Johansson (8) who found that the stationary assay, employing the *E. coli* mutant, was more suitable for routine estimations of vitamin B_{12} than the shaken assay. Aside from the economy of equipment and ease of preparation, the stationary assay appears to be less affected by extraneous factors, *e. g.*, thiamine.

Using *meta*-bisulfite extraction of samples, potency estimates obtained for vitamin B_{12} in multivitamin products by assay with the *E. coli* mutant, may be expected to coincide closely with those obtained by assay with *L. leichmannii*. However the presence of dried yeast or pseudo-forms of vitamin B_{12} may seriously affect both assays.

The precision of the modified *E. coli* assay has not been studied in great detail but it appears to be about the same as for the *L. leichmannii* assay (3). The response curve is stable and linear on an arithmetic plot. Departures from linearity have been rare and the slope-ratio method of calculation (16) has been found applicable to this assay. Since a stable linear response is obtained it is obvious that

fewer doses of standard and test preparations are required than for most other microbiological assays.

Although the U. S. P. procedure with *L. leichmannii* is widely accepted for the determination of vitamin B_{12} in liver extracts, pharmaceutical preparations, and natural materials, its disadvantages are well known. By comparison, the *E. coli* 113–3 assay is relatively simple to use, and can be applied to the estimation of vitamin B_{12} in a wide range of pharmaceutical products. It has not been found suitable for the assay of certain crude fermentation products or for preparations containing methionine. However, in view of its inherent advantages, the *E. coli* assay seems particularly applicable to the estimation of vitamin B_{12} activity in pharmaceuticals which do not contain interfering materials.

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The Age of Multivitamin Products on the Retail Market*

By J. A. CAMPBELL

A survey of the age of multivitamin products on the retail market was carried out in 21 centers in Canada. Lot numbers of all packages available for sale were listed for six of the larger manufacturers. Fifty per cent of the 9,260 samples examined were more than one year old and over 20 per cent were more than two years old. Thirty-eight samples were ten to fifteen years old. The data emphasize the need for better merchandizing control and point to the possible advantages of the use of an expiration date to achieve this.

RECENT WORK in this laboratory (1) indicated that the potency of some vitamins in multivitamin preparations was markedly below labeled claim one year after manufacture. About one-third of the products which were purchased at random on the retail market were older than one year. However, estimates of age of products on the retail market by some companies suggested that the average age of their products on the retail shelf was not more than six months and that only the occasional sample which was older than eighteen months would be found on the market. Discussions with a few druggists seemed to confirm this estimate. Since shelf life is so critical in the maintenance of potency, there appeared to be a need for more exact information on the actual age of representative preparations on the market. It is the purpose of this paper to present the results of a survey of the age of products in retail drug stores in cities of average size in Canada.

PROCEDURE

The survey was carried out on the products of six of the larger manufacturers in 21 cities across Canada. The locations are listed in Table I. All information was obtained during the month of December 1954. Food and Drug Inspectors in these areas were requested to list the lot numbers of all products containing three or more vitamins along with the number of packages of each. The size of package was disregarded. Information was sought on all capsules, tablets, and liquids available for sale in at least one large, one medium, and one small store in each area. The judgment of the store size was based on estimated prescription business and was left to the discretion of the individual inspector. It was suggested that it would interfere

* Received June 21, 1955, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.
The author acknowledges the generous assistance of many individuals without whose cooperation this study would not have been possible. He is indebted to the Food and Drug Inspectors in the 21 centers surveyed for examining drug stocks and listing the lot numbers; to more than 250 druggists for their assistance in the examination of their stocks, and to the six companies who furnished the date of manufacture of each lot number obtained.

TABLE I

Cities Surveyed			
1.	Victoria, B. C.	12.	Belleville, Ont.
2.	Edmonton, Alta.	13.	Ottawa, Ont.
3.	Calgary, Alta.	14.	Three Rivers, P. Q.
4.	Regina, Sask.	15.	Sherbrooke, P. Q.
5.	Saskatoon, Sask.	16.	Quebec, P. Q.
6.	Brandon, Man.	17.	Halifax, N. S.
7.	Port Arthur, Ont.	18.	Sidney, N. S.
8.	Windsor, Ont.	19.	Saint John, N. B.
9.	London, Ont.	20.	Charlottetown, P. E. I.
10.	Kitchener, Ont.		
11.	Hamilton, Ont.	21.	St. John's, N. F.

less with the business of an individual druggist if information was sought on only one or two companies from each one. In addition, it was requested that the inspector obtain the druggist's estimate of the approximate age of the products surveyed. Information was also obtained on storage conditions of multivitamin products in each store and on the druggist's source of products—i. e. wholesaler or direct from manufacturer.

The lot numbers obtained in this manner were decoded either by the manufacturer or by the author using the manufacturer's code. In each case there was a small percentage of lot numbers which could not be decoded for a variety of reasons including illegibility and errors in reading or transferring. In the case of one company (E) complete records were not available and it was impossible to determine the date of manufacture for many of the older products.

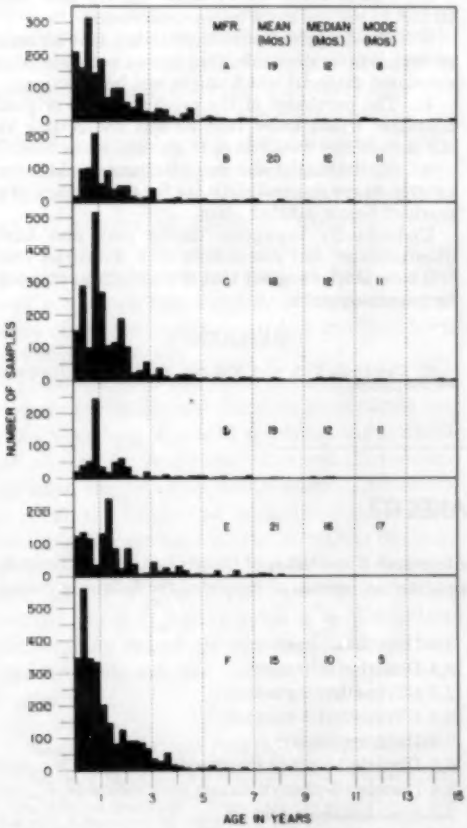

Fig. 1.—Frequency distributions of age of multivitamin products for six companies.

RESULTS AND DISCUSSION

The distribution of the age of samples of multivitamin products manufactured by six of the larger Canadian companies is shown graphically in Fig. 1. While similar in general shape, the distributions of various companies exhibit certain important differences. The mean age of products varied from 15 to 21 months. The median, which is the midpoint of the range, was similar in four cases but varied in two others. The mode, which is probably the best criterion for characterizing this type of information, varied from five months to seventeen months. It indicated that the peak of the distribution i. e. the most frequent age of product varied markedly from one manufacturer to another. In general, Company F had the best age distribution of products. Company A had a similar distribution but was not as effective in clearing old samples from the market. There was a lag of three to nine months before most products of companies B, D, and E reached the market. Company E, on which information was lacking on a number of old products, appeared to have a poor recall system. Some samples of Companies A and C were manufactured as far back as January and February, 1940.

In order to visualize the data from the standpoint of effective control, and to place companies on the same basis, it was important to know what proportion of products exceeded a certain age. The data, calculated in this manner are shown in Table 2. There were marked differences between companies in the proportion of products which were under six months old. Of the 9,260 samples examined 50% were older than one year. At this time or even before, it had been shown (1) that some vitamins were significantly below labeled claim. About 22% of the samples examined were older than two years, at which time some vitamins were shown to have been largely destroyed.

The data as a whole indicate that the preliminary estimate of age of samples on the retail druggists' shelf reported previously was on the conservative side. They give no substantiation to estimates of some companies who feel that their products have an average age of six months. While it is possible that individual companies may have better stock control than these figures indicate, other companies may be expected to have poorer control. None of the distributions have sufficiently sharp peaks and there are too many products in the two to six year age bracket. These factors point to a need for better merchandizing control on the part of all companies.

The data were collected from over 250 pharmacies. The size of drug store had little to do with the age of samples but there was a tendency for the smaller stores to have a higher proportion of older products. When asked how long they had had their stock, most pharmacists indicated that they attempted to turn their stock over every three to six months. Occasionally a druggist indicated

TABLE II.—DISTRIBUTION OF AGE OF PRODUCTS

Company	Per cent of Products							Number of Samples
	Under 1/2 yr.	Over 1/2 yr.	1 yr.	2 yr.	3 yr.	5 yr.	10 yr.	
A	21.8	78.2	51.2	24.5	12.9	3.4	1.0	1,710
B	13.5	86.5	50.5	23.4	13.5	4.4	0.1	868
C	20.7	79.3	50.5	18.7	9.1	3.4	0.7	2,140
D	9.2	90.8	45.3	20.2	10.4	3.5	0.2	653
E	21.0	79.0	66.8	27.9	16.7	4.7	0	1,221
F	34.1	65.9	40.4	19.7	7.3	1.5	0.1	2,668
Total	—	—	—	—	—	—	—	9,260
Weighted Means	23.3	76.7	49.5	21.8	10.8	3.1	0.4	—

that he had had a sample for two to five years or even ten years. In all cases it was noted, however, that there was a marked discrepancy between the pharmacist's estimate of the time he had had the product and the actual age of the product as determined from the lot number. Obviously such discrepancies could represent either time elapsed during handling by wholesaler and jobber or an over-conservative estimate of age on the part of the pharmacist. Whatever the cause, the important point was that the pharmacist evidently did not know the age of stock which he was selling. When stability is so critical, this situation could have serious consequences and would appear to point to the need for better stock control on the part of the druggist. However, unless he dated or coded all samples as he received them, he would have no means of determining the age of the product sold. Such a system would take no account of the lag which occurred between manufacture and distribution to the druggist.

Complete information on the druggists' source of vitamin products was not obtained in all areas. There was however, a definite indication that most products of Company F and many of Companies A and C were purchased directly from the manufacturer. Most products of Companies B, D, and E were purchased through wholesalers. Thus it appeared that direct sale to the druggist was effective in increasing the proportion of newer products on the retail shelf.

Storage conditions for the ordinary multivitamin preparations were listed as good or satisfactory in almost all stores. In a few, the druggist was advised to move his stock away from a source of heat. There appeared to be no provision for refrigerator storage of those liquid multivitamin products which were so labeled. Higher temperatures are known to hasten decomposition of vitamins in these products.

It is suggested that the use of an expiration date on multivitamin products, based on their known stability, would have several advantages.

1. There should be no products on the market which had passed their expiration date. This would tend to eliminate the situation described in this paper.

2. The druggist would know exactly how long he had to sell the stock he was purchasing.

3. The druggist would know, when making up a prescription or other sale, that he was not dispensing out-dated material which might not be effective.

4. The purchaser of the product in the original container would know that he was not getting an old sample and would have more confidence in it.

5. Both druggist and manufacturer would know exactly where responsibility lay for the potency of a product below labeled claim.

Undoubtedly expiration dating may also have disadvantages but discussions with industrial control laboratories suggest that the difficulties may not be unsurmountable.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation of Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

3-(Octadecyloxy)-1,2-propanediol (Batyl alcohol)
1,3,5-Trivinyl benzene
Technecium chloride
Trinitrosophloroglucinol
2-(*n*-Butyl)pyridine
1-Octadecylpyridinium chloride
2-Methylglutamic acid
Cholesterase
2,4-Dimethyl-1-hexene
2,4-Dimethyl-2-hexene
3,4-Dimethyl-1-hexene
3,4-Dimethyl-2-hexene
2,3,4-Trimethyl-2-pentene
3,4,4-Trimethyl-1-pentene
3,3-Dichloropropene
4,5-Dimethyl-*o*-phenylenediamine
2,4-Diamino-5-phenylthiazole hydrobromide
9-Amino-nonanoic acid
α-Amyrin (α-Amyrenol)
Eriochrome cyanine

A Quantal Method for the Study of Tolerance to Analgetics*

By FRED T. GALYSH†, ARTHUR TYE, and JOHN W. NELSON

A quantal modification of a thermal analgesimetric procedure was applied to the study of tolerance in albino rats. Four potent narcotic analgetics, morphine sulfate, methadone hydrochloride, metopon hydrochloride, and levorphan tartrate, were administered daily for seven weeks and the amount of each drug required to produce a chosen degree of analgesia, the AD_{50}, determined at weekly intervals. Analysis of the data so obtained indicated that the rate of tolerance development is different for each of these four drugs. The rate was most rapid with levorphan, while metopon, methadone, and morphine showed lesser rates. The results seem to agree reasonably well with clinical impressions and the procedure is suggested as a simple and reliable method for the measurement of tolerance development to analgetics in animals.

A WELL-RECOGNIZED DRAWBACK to the use of opiates and most synthetic analgetics in the treatment of chronic pain is the development of tolerance to their analgesic action. Moreover tolerance to such drugs is generally associated with addiction so that information on tolerance-development may well be of value in predicting addiction liability. The establishment of an accurate, reproducible, and practical quantitative pharmacological method suitable for the study of analgesic tolerance phenomena in animals would, therefore, seem desirable.

One of the most satisfactory techniques for the study of analgesia is the utilization of radiant heat stimuli. Thermal radiation methods or modifications thereof have been employed in several studies of analgesic tolerance (1–5). Such methods have usually depended either upon measurement of the intensity of heat applied over a constant exposure time or upon measurement of the reaction time with a constant level of thermal intensity.

These methods have not been entirely satisfactory, partly because the data so obtained are often a mixture of graded responses and quantal (all-or-nothing) responses. Tye and Christensen (6) have suggested an analgesimetric procedure in which the AD_{50} of an analgetic is determined by using radiant heat as the stimulus, the skin of the rat tail as the receptive field and the tail-flick as the response to the stimulus. This method avoids mixed responses and therefore yields results of a purely quantal nature. It was therefore selected for use in these tolerance studies.

* Received April 22, 1955, from the Ohio State University, College of Pharmacy, Columbus 10, Ohio.

† Fellow of The American Foundation for Pharmaceutical Education.

The authors gratefully acknowledge the assistance of the Statistics Laboratory of The Ohio State University in analysis of the data included in this publication.

The authors wish to thank Hoffmann-La Roche, Inc., for supplying the levorphan tartrate used in this study.

EXPERIMENTAL

The animals employed were males and females of the Wistar strain of albino rat. They were housed and fed under uniform conditions and maintained on a diet of Purina Laboratory Chow and water *ad libitum*.

The drugs studied were morphine sulfate, methadone hydrochloride, metopon hydrochloride, and levorphan tartrate.

Aqueous solutions were adjusted so that the volume administered was between 0.2 and 1.0 ml. All injections were made subcutaneously with a 1.0-ml. capacity tuberculin syringe graduated to 0.01 ml.

Equal numbers of male and female animals were used in each dose group in order to neutralize any variation due to sex. To study the effect of age on development of tolerance, two groups of rats were used in conjunction with each analgetic agent studied. The initial weights of the rats in one group ranged from 120 to 140 Gm.; those in the second group weighed from 150 to 170 Gm. The age difference between the two groups was approximately four weeks. Both of these groups, each consisting of 36 rats, were subdivided into three dose groups. Each dose group was therefore comprised of 6 male and 6 female rats. A similar group was used as a control for each drug by injecting each animal with an amount of sodium chloride equivalent to the amount of analgetic administered to the test rats.

Low, medium, and high doses of each analgetic were injected into successive dose groups which were then exposed, after a period of forty minutes, to a radiant heat stimulus of 320 millicalories/second/square centimeter for three seconds. This stimulus should cause a pain of about 5 or 6 "dols" which approximates the degree of pain that narcotic analgetics are generally called upon to overcome clinically. Failure to respond by moving the tail was regarded as analgesia. The percentage of each dose group showing analgesia was plotted against the dose on logarithmic probability paper. From the curve so obtained the dose of the analgetic that would cause analgesia in 50 per cent of the test animals was read (AD_{50}).

The Wilcoxon and Litchfield (7) graphical method was used for determining whether a straight line fitted the plotted points satisfactorily and for estimating the AD_{50} with 19/20 confidence limits.

The AD_{50} was determined for a drug at the outset. This determination was repeated at least twice at three-day intervals in order to establish firmly an initial AD_{50}. This AD_{50} was then administered to the animals as a single daily dose for one week. The AD_{50} was redetermined on the first day of the second week. This new AD_{50} was then administered daily throughout the second week; a new AD_{50} determined on the first day of the third week, and so on. The experiment, as originally planned, was to continue for twelve weeks. It was found, however, that symptoms of toxicity began to appear about the sixth week. Therefore, the experiment was discontinued at the end of the seventh week.

In Tables I and II are shown the weekly AD_{50} determined for each analgetic over the seven-week period of chronic administration.

TABLE I.—AD_{50}[a] AS TOLERANCE DEVELOPS IN YOUNGER ANIMALS

Time	Morphine	Methadone	Metopon	Levorphan
0	2.8	2.0	0.40	0.24
1	4.2	2.6	0.56	0.36
2	4.8	3.0	1.4	0.62
3	8.4	3.4	1.6	0.91
4	8.0	3.6	2.3	1.4
5	7.0	5.1	2.7	3.4
6	13.9	7.4	3.7	6.6
7	18.0	5.5	3.3	7.2

[a] AD_{50} is given in mg./Kg. and time in weeks.

TABLE II.—AD_{50}[a] AS TOLERANCE DEVELOPS IN OLDER ANIMALS

Time	Morphine	Methadone	Metopon	Levorphan
0	3.6	1.8	0.39	0.24
1	4.2	1.9	0.67	0.23
2	5.2	2.6	0.96	1.0
3	8.2	3.3	1.6	0.91
4	6.5	4.1	2.1	1.1
5	10.0	4.3	3.0	2.9
6	8.0	11.6	3.0	5.3
7	9.9	8.2	3.3	6.7

[a] AD_{50} is given in mg./Kg. and time in weeks.

As can be seen from the tables the AD_{50} for each drug showed a marked tendency to increase during the period of chronic administration. It would seem reasonable to consider this rise as a measure of the development of tolerance to a drug.

It was realized that this apparent increase in the AD_{50}, which was measured in all cases forty minutes after injection of the drug, might be due to a change in the peak action time of the drug. To rule out this possiblity the following experiment was performed.

A mixed group of 10 rats was used for each of the drugs. The AD_{50} of the drug being tested was administered daily as in the original experiment and the percentage of rats exhibiting analgesia at 20, 40, and 60 minutes post-injection measured each week for seven weeks. It was found that the peak-action time for each of the drugs remained constant around forty minutes post-injection. It seems, therefore, that the increase in the AD_{50} as measured in the original experiments reflected a true decrease in analgesic effect rather than an increase or decrease in time of analgesic onset.

RESULTS AND DISCUSSION

Most previous studies of analgesic tolerance have measured the decrease in effect produced by repeated administration of a fixed dose of a drug. In this study the increasing dosages necessary to protect against a fairly severe degree of pain were determined over an extended period. This would seem to be more analogous to the actual clinical problem. To heighten this analogy, the daily dose was not fixed but was changed each week in accordance with the dose required to produce the original effect, as would be necessary clinically.

The development of tolerance, then, was evidenced by a progressive increase in the AD_{50} when measured at weekly intervals during a period of daily administration.

To determine whether tolerance develops at the same rate for each drug, the following statistical procedure was employed.

For each drug and each week,

$$\log_e \frac{AD_{50}}{\text{Initial } AD_{50}} = y$$

was calculated. A "least square" line of the form $y = at$ was obtained for each case (t = time and a = slope). Then, estimates of the variance about the "least square" line were obtained. Summaries of this data appear in Tables III and IV.

TABLE III.—ESTIMATES OF "a"

	Morphine	Methadone	Metopon	Levorphan
Younger animals	0.26	0.18	0.37	0.50
Older animals	0.16	0.23	0.36	0.48

TABLE IV.—ESTIMATES OF THE VARIANCE ABOUT THE "LEAST SQUARE" LINE

	Morphine	Methadone	Metopon	Levorphan
Younger animals	0.0391	0.0198	0.0924	0.0325
Older animals	0.0299	0.0534	0.0648	0.1027

The ratios of the differences between the "a's" for different pairs of drugs to their standard deviations are shown in Table V. The hypothesis hereby tested was that the slopes (and therefore the rate at which tolerance develops) for each pair of drugs are the same. A ratio value greater than 2.58, obtained by dividing the differences between the "a's" for different pairs of drugs by their standard deviations, was considered significant, since according to a table of normal distribution (8) the probability of obtaining a greater value is less than one per cent.

Ratios of the differences between "a's" for different ages to their standard deviations were also calculated (Table VI).

From this statistical data, it may be concluded that the rate at which tolerance develops is significantly different ($P = 0.01$) in the four drugs. The rate at which tolerance develops is not significantly different between the younger and older animals except in the case of morphine.

A quantitative relationship between the rates of

TABLE V.—RATIOS OF DIFFERENCES BETWEEN "a's" FOR DIFFERENT PAIRS OF DRUGS TO THEIR STANDARD DEVIATIONS

	Methadone	Metopon	Levorphan
	Younger Animals		
Morphine	4.03	−3.61	−10.78
Methadone	...	−6.82	−16.91
Metopon	...	...	− 4.45
	Older Animals		
Morphine	−2.73	−7.69	−10.33
Methadone	...	−4.58	− 7.53
Metopon	...	...	− 3.42

TABLE VI.—RATIOS OF DIFFERENCES BETWEEN "a's" FOR DIFFERENT AGES TO THEIR STANDARD DEVIATIONS

Morphine	Methadone	Metopon	Levorphan
−4.34	2.31	−0.21	0.70

tolerance development can be obtained by comparison of the slopes, "a's", of the "least square" lines for the four drugs (Table VII and Figs. 1 and 2).

In the younger animals levorphan appears to show the most rapid rate of tolerance development with metopon, morphine, and methadone following in

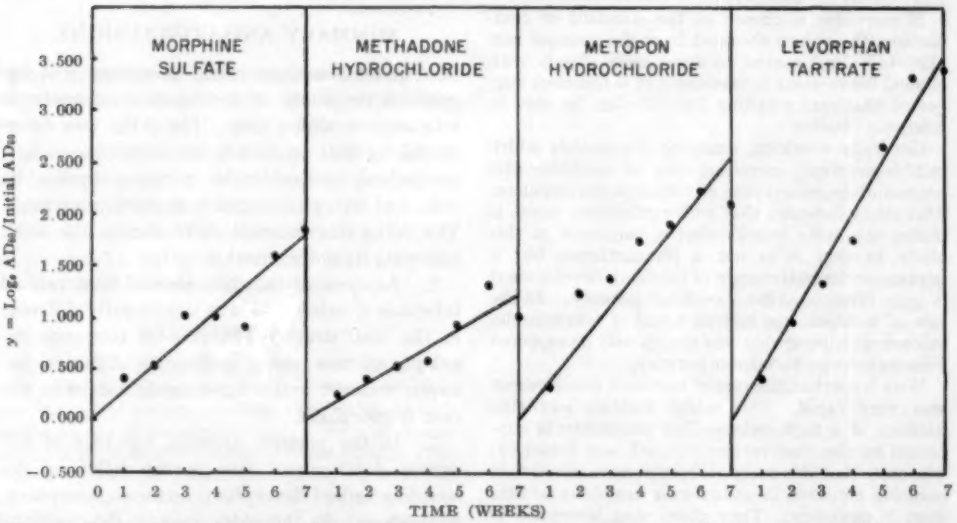

Fig. 1.—Comparisons of rates of tolerance development in the younger rats. The curves for each drug represent the "least square" lines of the form $y = at$.

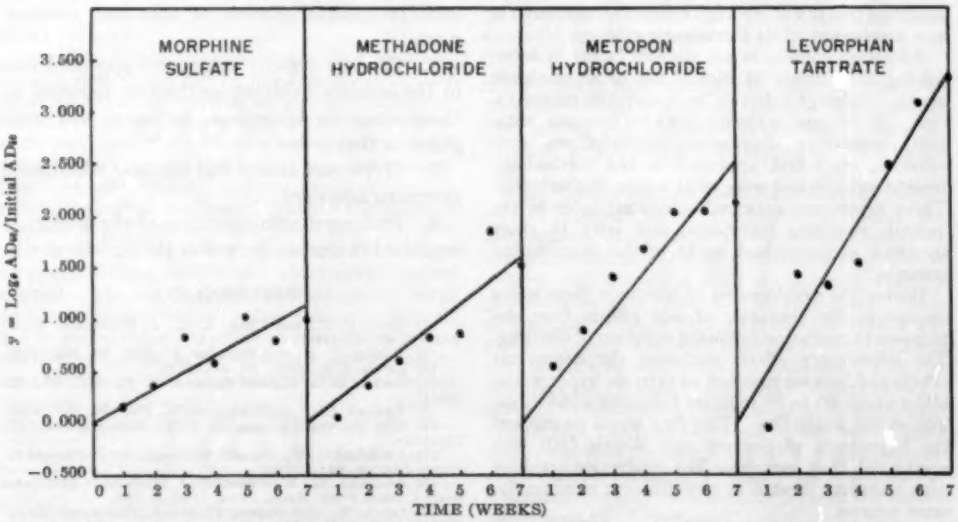

Fig. 2.—Comparisons of rates of tolerance development in the older rats. The curves for each drug represent the "least square" lines of the form $y = at$.

TABLE VII.—COMPARISON OF RATES OF TOLERANCE DEVELOPMENT RELATIVE TO MORPHINE (YOUNGER ANIMALS) = 1.0.

	Morphine	Methadone	Metopon	Levorphan
Younger animals	1.0	0.69	1.4	1.9
Older animals	0.62	0.88	1.4	1.8

that order. In the older animals the slopes are essentially the same except with morphine where the rate of tolerance development shows a significantly lower value. A possible explanation of this finding is that, in the case of morphine, enzyme systems of the older animals are less adaptable and develop more slowly the ability to form inactive congeners.

If morphine is chosen as the standard of comparison, the values obtained from the younger rats (120–140 Gm.) appear to agree more closely with clinical impressions in humans. It is therefore suggested that rats weighing 120–140 Gm. be used in tolerance studies.

Generally speaking, analgetic compounds which exhibit potencies exceeding that of morphine also possess an increased rate of tolerance development. This study indicates that with methadone, which is unlike the other two synthetics employed in this study in that it is not a phenanthrene but a heptanone derivative, rate of tolerance development is quite dissociated from analgesic potency. In the case of metopon, the increased rate of tolerance development is somewhat less than would be expected from its increased analgesic potency.

With levorphan the rate of tolerance development was very rapid. This might indicate addiction liability of a high order. This possibility is supported by the observations of Isbell and Fraser (9) who state that this analgetic is even more effective in inducing euphoria in nontolerant morphine addicts than is morphine. They claim that levorphan is very efficient in relieving or suppressing the symptoms of abstinence from morphine in man. It may be pointed out, however, that the extremely high analgesic potency of levorphan must be considered in any assessment of its therapeutic efficacy.

Addiction liability is not the sole factor in determining the efficacy of clinical use of an analgesic agent. Relative safety is an important consideration. Symptoms of chronic toxicity (extreme sedation, respiratory depression, exophthalmos, convulsions, etc.) first appeared in the methadone-treated animals and were most severe in this group. These symptoms appeared somewhat later in the animals receiving levorphan, still later in those receiving metopon and lastly in the morphinized animals.

During the development of tolerance there was a change in the character of side effects from depression to excitation following injection of the drug. The stimulatory effects outlasted the depressant effects and became manifest as extreme hyperexcitability about 60 to 90 minutes following administration of the analgetics. This fact seems to support the hypothesis of Seevers and Woods (10) who postulated that morphine-like analgetics combine with receptors located at two different sites on the same neuron.

Statistically significant differences in the mean increase in body weight of the test animals and controls indicated that the analgesic compounds employed in this study retarded growth of rats. Analysis of the data showed that levorphan had little effect on growth of the group of older rats.

After discontinuance of the drugs an attempt was made to observe withdrawal symptoms. The only symptom observed was hyperexcitability, which reached its maximum in 24 to 48 hours.

Due caution must be used when attempting to correlate the findings of a study such as this with effects in man. It seems reasonable, nonetheless, to suggest that such determinations, considered in conjunction with estimations of analgesic potency, may prove useful in structure-action studies and the search for potent analgetics with low addiction and tolerance capacities.

SUMMARY AND CONCLUSIONS

A quantal analgesimetric procedure was applied to the study of development of analgetic tolerance in albino rats. The AD_{50} was determined by this procedure for morphine sulfate, methadone hydrochloride, metopon hydrochloride, and levorphan tartrate at weekly intervals. The AD_{90} was injected daily during the week following its determination.

1. Analysis of the data showed that rate of tolerance development was significantly different in the four drugs. The rate of tolerance development was not significantly different between younger and older animals except in the case of morphine.

2. In the younger animals, the rate of tolerance development was in the following descending order: levorphan, metopon, morphine, methodone. In the older animals the positions of morphine and methadone were reversed.

3. Rats weighing 120–140 Gm. should be used in quantal studies of tolerance development.

4. Chronic toxicity symptoms appeared first in the animals receiving methadone followed by those receiving levorphan, metopon, and morphine in that order.

5. Hyperexcitability was the only withdrawal symptom observed.

6. Prolonged administration of the analgetics employed in this study retards the growth of rats.

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A Biological Method for the Evaluation of the Nonnarcotic Analgesics*

By R. D. GIBSON†, T. S. MIYA, and L. D. EDWARDS‡

A procedure is described for the biological evaluation of nonnarcotic analgesics in rats. The apparatus employed consisted of a contact rectal electrode, and a copper sheet electrode in contact with all four feet, and a graded electrical stimulus. The minimal effective doses and their relative analgesic potencies as compared to acetylsalicylic acid are as follows: acetylsalicylic acid, 450 mg./Kg., 1.0; acetophenetidin, 750 mg./Kg., 0.6; sodium salicylate, 600 mg./Kg., 0.75; aminopyrine, 450 mg./Kg., 1.0; sodium acetylsalicylate, 150 mg./Kg. (dose calculated on the basis of acetylsalicylic acid), 3.0; acetanilid, 150 mg./Kg., 3.0; salicylamide, 75 mg./Kg., 6.0; antipyrine, 75 mg./Kg., 6.0, all administered orally, and morphine sulfate, subcutaneously, 5 mg./Kg., 90.

THE NEED for an adequate screening method for nonnarcotic analgesics has been realized by researchers for many years. Much work has been done in this field with no agreement among the workers as to the validity of the results obtained by any existing method.

This is a report of the results obtained ultilizing an electrical rectal stimulating apparatus for the evaluation of nonnarcotic analgesics.

EXPERIMENTAL

Apparatus.—The apparatus consisted of a 10 x 10 x 6-inch plastic container with a thin sheet (0.25 mm. thick) of copper covering the bottom, which served as one of the electrodes. A piece of copper tubing approximately 3/4-inch long and 1/4-inch wide, shaped to resemble a large fecal dropping served as the rectal electrode. The 110-volt line current was varied by an E^{86} Volt Stimulator, Type R (Gorrell & Gorrell, Haworth, New Jersey). The voltage was read to the nearest 0.1 volt from an Electronic Voltmeter Ohmmeter, Model 100 (Electronic Design, Inc., New York), connected in parallel in the circuit (Fig. 1).

Procedure.—Adult female albino Sprague-Dawley rats were fasted for twenty-four hours. After removing the fecal material by gentle digital pressure, 1 to 2 cc. of normal saline solution was placed in the rectum. The contact electrode was then inserted approximately 1 1/2 inches into the rectum. The electrode was held in place by attaching the protruding wire to the base of the rat's tail with adhesive tape and a small artery clamp (Fig. 1). The rat was then placed on the copper sheet which had previously been covered with 1/8-inch normal saline solution. The rat's tail and the lead to the rectal electrode were gently held by hand in a vertical position to prevent shorting of the circuit. The current was applied for one to two seconds at one second intervals at every voltage setting. The voltage was increased by 0.1-volt increments until a squeak response was obtained. The animal was stimulated

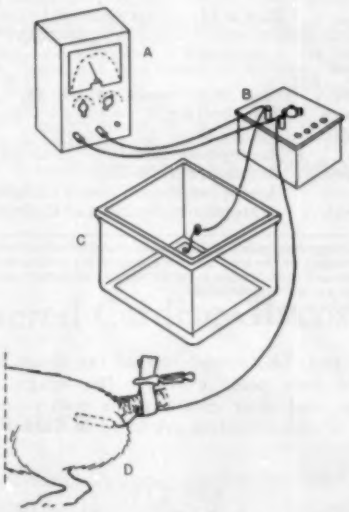

Fig. 1.—Apparatus employed. A—voltmeter; B—E^{86} volt stimulator; C—plastic container with copper sheet electrode; D—detail of insertion.

three times at each voltage and a squeak two out of three times was considered to be the end point. This voltage was designated as the reaction voltage. The normal reaction voltage ranged from 0.5 to 2.5 volts.

The reaction voltages were determined at thirty-minute intervals for a period of three hours; the first reading being taken thirty minutes after the administration of the drug. A four-day cross-over pattern was employed with six animals, thereby providing data on 12 animals for each dose. Doses of the various drugs were administered until a significant difference in peak reaction voltages between control and treated animals was observed. This was determined by the use of the Fisher "t" test at 11 degrees of freedom using a one-tailed 95% probability (Table I). The peak reaction voltages as employed in this test were the means of the two highest successive reaction voltages.[1] No attempt was made to determine duration of action, because of the nature

* Received February 21, 1955, from the Research Laboratories, Purdue University School of Pharmacy, Lafayette, Ind. Presented at the AAAS meetings Dec. 1954, Berkeley, Calif.

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‡ Deceased.

[1] We are indebted to the Statistical Department, Purdue University, for aid in the experimental design.

TABLE I.—SUMMARY OF THE MINIMUM EFFECTIVE DOSES OF NONNARCOTIC ANALGESICS IN RATS

Drugs[a]	Minimum Effective Dose, mg./Kg. (oral)	Mean Peak Voltages[b] Treated	Controls	Peak Effect in Min.[c]
Acetylsalicylic Acid	450	2.12 ± 0.20[d]	1.35 ± 0.15	60–90 0.01
Sodium Acetylsalicylate	150[e]	1.78 ± 0.16	1.26 ± 0.19	60–90 0.05
Sodium Salicylate	600	2.25 ± 0.11	1.43 ± 0.12	60–90 0.005
Salicylamide	75	2.26 ± 0.14	1.78 ± 0.10	90–120 0.025
Acetanilid	150	1.95 ± 0.18	1.24 ± 0.12	90–120 0.005
Antipyrine	75	1.72 ± 0.21	1.38 ± 0.21	30–60 0.05
Aminopyrine	450	1.67 ± 0.14	1.20 ± 0.14	60–90 0.05
Acetophenetidin	750	1.87 ± 0.16	1.33 ± 0.17	30–60 0.05
Morphine SO_4	5 (subc.)	2.84 ± 0.17	1.71 ± 0.21	30–60 0.01
Untreated		1.45 ± 0.09	1.41 ± 0.08	No sig. diff.

[a] Suspended in 10% acacia, except morphine.
[b] The mean of the two highest consecutive voltages of each animal in the group was averaged to obtain mean peak voltages.
[c] Time and significance level at which difference occurred (Fisher "t" one-tailed probability at 11 d. f.).
[d] Standard error.
[e] Dose calculated on the basis of acetylsalicylic acid.

TABLE II.—ANALGESIC STUDIES OF DRUG COMBINATIONS IN THE ALBINO RAT[a]

Acetylsalicylic Acid, mg./Kg.	Drug(s) and Dose, mg./Kg.	Mean Peak Voltages[b] Treated	Controls	Peak Effect in Min.[c]
150 with	PABA[d] 150	1.61 ± 0.19[e]	1.58 ± 0.19	No sig. diff.
300 with	PABA 300	1.93 ± 0.16	1.78 ± 0.13	No sig. diff.
150 with	Acetophenetidin 100	1.73 ± 0.11	1.96 ± 0.17	Controls higher
300 with	Acetophenetidin 220	2.41 ± 0.16	1.72 ± 0.18	30–60 0.005
150 with	Acetophenetidin 110 and Caffeine 22	1.68 ± 0.09	1.92 ± 0.09	Controls higher
300 with	Acetophenetidin 220 and Caffeine 44	1.29 ± 0.14	1.30 ± 0.19	Controls higher

[a] All drugs suspended in 10% acacia and administered orally.
[b] The mean of the two highest consecutive voltages of each animal in the group was averaged to obtain mean peak voltages.
[c] Time and significance level at which difference occurred (Fisher "t" one-tailed probability at 11 d. f.).
[d] *Para*-aminobenzoic acid.
[e] Standard error.

of the test, i.e., comparison of the drugs at the time of their peak activity. The drugs, doses, vehicles, and drug combinations tested and the routes of administration are listed in Tables I and II.

RESULTS

Sodium salicylate and acetylsalicylic acid were found to be devoid of analgesic activity when suspended in 1% sodium alginate solution. However, both sodium salicylate (600 mg./Kg.) and acetylsalicylic acid (450 mg./Kg.) were effective when suspended in 10% acacia. Subsequent investigation in this laboratory indicates that both of these drugs are effective when suspended in sodium alginate of lesser concentration.

Para-aminobenzoic acid did not appear to potentiate the analgesic activity of acetylsalicylic acid or sodium salicylate.

A combination of 300 mg./Kg. of acetylsalicylic acid and 220 mg./Kg. of acetophenetidin produced a significant increase in reaction time. Caffeine alkaloid 44 mg./Kg. abolished this action (see Table II). Oral administration of 25 and 50 mg./Kg of caffeine alkaloid increased the sensitivity of the animals to the electrical stimulus. This would seem to indicate that the incorporation of caffeine in certain analgesic preparations is unwarranted. Subcutaneous injection of 2.5 mg./Kg. of atropine sulfate was found to have no detectable analgesic activity.

The peak reaction voltages (means of the two highest successive squeak threshold voltages) were in most cases between 60–90 minutes (Tables I and II) which appears to have a direct correlation to the peak salicylate blood levels after oral administration reported for humans (1–3) and animals (4, 5).

The relative analgesic potencies of each drug suspended in 10% acacia were determined by comparing the minimum effective doses of each analgesic to acetylsalicylic acid.

The relative analgesic potencies of the drugs were as follows: acetylsalicylic acid, 1; acetophenetidin, 0.6; sodium salicylate, 0.75; aminopyrine, 1; sodium acetylsalicylate (dose calculated on the basis of acetylsalicylic acid), 3; acetanilid, 3; salicylamide 6; antipyrine, 6; and morphine sulfate, 90.

DISCUSSION

The stimulating apparatus, simple in design and application, compares favorably with any procedure reported for laboratory animals. The threshold to electrical stimulation closely approximated that reported for an isolated nerve (6). The voltages employed in this investigation to produce a squeak response are considerably lower than those reported in mice (7) or rats (8). This method also possesses all of the desirable characteristics proposed by Goetzl, *et al.* (9), with the exception that it was not "applicable to isolated pain receptors."

The procedure afforded several advantages over some of the other available testing methods. The tail flick of the rat and skin twitch of the guinea pig have been shown to exist when spinal animals are subjected to a thermal stimulus (10). A squeak response which involves the participation of the higher centers affords a better criterion of the reaction to pain and is therefore more desirable.

The cross-over technique affords data that can be easily evaluated statistically and eliminates the procedure of selecting specific animals which is necessary with the radiant-heat method of D'Amour and Smith (11).

A possible limitation of this procedure was its failure to detect moderate doses of the nonnarcotic analgesics. In view of the inability to detect low doses of these drugs by other testing methods, it appears plausible that psychological factors which may play a role in the alleviation of pain in humans cannot be measured by these methods in laboratory animals.

It is interesting to note that caffeine which is incorporated in certain nonnarcotic analgesic preparations appeared to abolish the analgesic effects of acetylsalicylic acid, whereas acetophenetidin and acetylsalicylic acid appeared to potentiate each other.

SUMMARY

A method for the analgesic evaluation of certain compounds by use of a rectal electrode and an electrode in contact with all four feet which employs the squeak response in albino rats is presented. The voltage required to induce a squeak is considerably smaller than that previously reported. The interval at which time a significant difference in squeak response threshold between control and treated animals occurred appeared to coincide with the peak blood salicylate levels after oral administration reported by other workers.

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Absorption of Orally Administered Cardiac Glycosides in Cats*

By WALLACE F. WHITE

Digitoxin, gitalin, gitoxin, and tinctures of *Digitalis lanata, lutea, sibirica, orientalis,* and *purpurea* were administered by stomach tube to unanesthetized cats to obtain LD_{50} estimates of carditonic drugs. These drugs differ in their emetic action and extensive experimental data are presented.

CARDIAC GLYCOSIDE solutions have been injected intravenously into cats and other experimental animals in order to estimate the potency of the drugs as cardiotonic agents for human use. Clinically, however, these drugs are used chiefly by oral administration. Therefore, the matter of absorption from the intestinal tract is important. A previous preliminary study (1) indicated that glycosides differ in rate and extent of absorption when administered orally to cats. The present study uses a different method for estimating absorption, which is based upon the ratio between oral LD_{50} and average i. v. lethal dose. The cat was considered a suitable test animal especially since considerable work has been reported in the literature on i. v. testing in this species.

Both purified glycosides and galenical mixtures were studied in order to indicate whether all intravenously active constituents of *Digitalis purpurea* are absorbed to the same extent and whether other species of *Digitalis* are comparable to *purpurea* in absorption. The purified drugs chosen were those which have been identified as derivatives of *Digitalis purpurea;* namely, digitoxin, gitalin, and gitoxin. The crude cardiotonic drugs available were leaves of *D. purpurea, lanata, lutea, orientalis,* and *sibirica.* The correct identity of the last species mentioned needs confirmation, but the other species have been identified positively by Dr. E. B. Fischer, Professor of Pharmacognosy, University of Minnesota. The crude drugs were grown in the medicinal plant garden of the University under uniform conditions and the leaves were dried and powdered so that valid comparisons could be made between the five species of *Digitalis.* At appropriate times tinctures were made by mixing 10 cc. of an

* Received April 23, 1955, from the University of Minnesota, College of Pharmacy, Minneapolis 14.
This investigation was supported by research funds of the Graduate School of the University of Minnesota.

aqueous alcohol solution (1 part H_2O to 4 parts alcohol) with each gram of powder and shaking mechanically for 24±2 hours. After centrifuging and decanting, each cc. of tincture contained the extractable material from 100 mg. of dried leaf.

Of the purified drugs,[1] gitoxin was finely powdered in an agate mortar and placed in capsules with 19 parts of lactose; digitoxin and amorphous gitalin were dissolved in an aqueous alcohol solution as above.

EXPERIMENTAL

Healthy adult cats were accepted from all available sources. The average weight calculated from 100 cats selected at random was 2.29 Kg. The animals were fasted for 36 to 48 hours in order to provide a uniformly empty gastrointestinal tract. Except for seven uncooperative animals, all cats were tied down on their backs on animal boards without the use of anesthesia. The seven were tied under light ether anesthesia and then were permitted to recover for an hour before the experiment started. None of these seven cats vomited during the experiment; therefore, ether did not appear to alter the experimental conditions necessary to study absorption.

Drug solutions were administered by means of a hard rubber tube about 14 inches long, having an inside diameter of 3 mm. The assistance of an attendant was necessary to place the tube through the mouth into the stomach and have it retained long enough to insure that the dose was delivered in its entirety. Approximately 30 cc. of water was given as a rinse, and to dilute the alcoholic solution or tincture below the concentration that would be expected to produce local irritation. Gitoxin diluted with lactose was given in gelatin capsules and swallowing induced by small portions of water. This method, however, was found to be unsatisfactory for administering powdered leaf because many capsules (20 or more) had to be given and because the cats in a preliminary study had expelled part of the drug before swallowing. Tinctures made from the powdered leaves were very satisfactory and could be administered by stomach tube.

After oral administration of the drug each cat was retained on the animal board for a period of six and one-half hours in order to provide uniform conditions for absorption. If there was vomiting during this period the animal was not used in calculation of the LD_{50}. When vomiting did not occur, which was the usual case, the cat was removed from the animal board after six and one-half hours, placed in a cage, and kept under periodic observation for two days. The dosage-mortality curves were claculated on the basis of the per cent mortality at the end of forty-eight hours after oral administration at each dosage level. The forty-eight-hour end point was selected arbitrarily on the basis of experience obtained during the previous study (1).

[1] The author is indebted to Winthrop-Stearns Inc., New York, for the digitoxin (Win #2064) and for the gitoxin (V #59); and to White Laboratories Inc., Kenilworth, N. J., for the amorphous gitalin (Citaligin batch #23150 and R 24100).

RESULTS

In the statistical analysis of the data the pattern outlined by D. J. Finney (2) was followed. Table I is a summary of the data used in calculating LD_{50} estimates based upon oral administration. The individual regression curves were accurately represented as straight lines according to the chi-square test. The mean regression curve had a slope of 7.39. The odds are 90 in 100 according to the chi-square test ($\chi^2_7 = 2.80$) that the differences observed in the individual slopes of the 8 dosage-mortality curves could have arisen by chance alone if all were characterized by the mean slope. This indicates that differences in cardiotonic drug action were quantitative but not qualitative.

The LD_{50} estimates obtained from stomach tube administration were compared with average i. v. fatal doses in order to estimate per cent absorption. Comparisons could be made in every case except with gitoxin which failed to kill cats by oral administration but did kill by i. v. administration. By this method of estimating absorption, digitoxin was absorbed completely; gitalin, 85%; and gitoxin, not at all during the six and one-half hour investigation period. It is of interest to note that tincture of *Digitalis purpurea* containing, among other things, these 3 cardiotonic materials had active materials absorbed to the extent of 25%. The other tinctures, with the exception of that made from *Digitalis orientalis*, were absorbed to the extent of 28 to 31%. The poorest absorption was experienced with *Digitalis orientalis* in which 13% of the active materials were absorbed, which might indicate a greater proportion of gitoxin-like material in this crude plant material. In 70% alcohol control experiments, 6 cc./Kg. orally and 1 cc./Kg. i. v. every ten minutes failed to kill but did produce a stuporous condition lasting several hours. In the control experiments the cats were subjected to 3 or more times the quantity of alcohol given with cardiotonic drugs, summarized in Tables I and II.

Comparison of Tables I and II will call attention to the fact that the arrangement of drugs from high to low potency is not the same when the drugs are tested intravenously and orally.

In order to study absorption in a quantitative fashion it is necessary that vomiting be avoided for a reasonably long period of time. During the initial stage of this study it was found that most of the cats did not vomit at all if they remained immobilized on the animal boards after the drug was administered. A six and one-half hour period was selected arbitrarily to allow for absorption of the active materials from the intestinal tract. Since the cats had been fasted for thirty-six to forty-eight hours there was nothing to hinder absorption. It is not known whether all absorbable materials introduced were absorbed or not in the six and one-half hours, but it is possible to make comparisons since all drugs were treated under similar experimental conditions and since cats were discarded if vomiting occurred. The digitoxin data are pertinent in this connection since Table II indicates 100% absorption. Gold (4) has estimated that 100% of digitoxin is absorbed and 20% digitalis leaf is absorbed in humans when the drugs are given orally. My estimate for digitalis leaf in cats is 25% absorption, which likewise shows close agreement in these two species.

TABLE I.—CARDIOTONIC DRUGS ADMINISTERED BY STOMACH TUBE TO FASTED CATS (ANIMALS THAT VOMITED WERE NOT INCLUDED)

Drug	Dose, mg./Kg.	No. Died/ No. Injected	LD_{50} ± S.E., mg./Kg.	Fiducial Limits, LD_{50} ± 1.96 S.E.
Digitoxin	0.26	2/10		
	0.29	5/10		
	0.33	6/10	0.32 ± 0.07	0.23–0.43
	0.43	9/10		
Gitalin	0.97	1/10		
	1.12	5/11	1.23 ± 0.08	1.09–1.42
	1.30	8/10		
	1.50	6/11		
D. lanata Tr. (10%)				
Fresh Tr.	75.0	3/11		
	100.0	3/10	103.0 ± 12.2	59.0–179.0
	133.0	9/11		
Year-old Tr.	75.0	1/5		
	100.0	2/10	127.0 ± 10.7	78.0–206.0
	133.0	5/10		
	171.0	9/11		
D. sibirica Fresh Tr.	100.0	1/10		
	133.0	6/10	132.0 ± 9.1	87.0–198.0
	171.0	8/10		
D. orientalis Fresh Tr.	133.0	1/10		
	171.0	5/10	175.0 ± 8.8	118–258
	220.0	8/10		
D. lutea Fresh Tr.	150.0	1/10		
	193.0	5/10	203.0 ± 9.7	131–314
	250.0	7/10		
D. purpurea Fresh Tr.	150.0	1/10		
	193.0	3/10	244.0 ± 11.4	146–408
	250.0	6/10		
	322.0	7/10		

TABLE II.—ESTIMATED PER CENT ABSORPTION OF ACTIVE CARDIOTONIC SUBSTANCES FROM THE INTESTINAL TRACT OF CATS DURING A SIX AND ONE-HALF HOUR IMMOBILIZATION PERIOD

Drug	No. Cats Injected i.v.	Mean Lethal Dose, mg./Kg.	Oral LD_{50} / Mean i. v. LD × 100
Digitoxin[a]	4	0.31 ± 0.05[b]	100
Gitoxin[a]	3	0.66 ± 0.10[c]	0
Gitalin	5	1.04 ± 0.30[d]	85
D. orientalis			
Year-old Tr.	6	25.8 ± 4.67	...
Fresh Tr.	6	26.1 ± 11.03	13
D. lanata			
Fresh Tr.	6	29.9 ± 5.2	29
Year-old Tr.	6	36.4 ± 5.2	28
D. sibirica Fresh Tr.	6	36.6 ± 4.6	28
D. purpurea Fresh Tr.	6	62.0 ± 10.8	25
D. lutea Fresh Tr.	6	63.8 ± 7.7	31

[a] Dissolved in 20% alc. sol. and injected every ten min.
[b] K. K. Chen's estimate was 0.33 mg./Kg. (3).
[c] Rothlin's estimate was 0.73 mg./Kg. (5).
[d] Rothlin's estimate was 0.8 mg./Kg. (5) and Strand, *et al.*, 0.65 mg./Kg. (6). Since the material is amorphous, it is doubtful that the samples of drug were the same material in the 3 laboratories.

All cats were seen to vomit or attempt to vomit shortly after being freed from the animal boards. Retching usually continued for several days in surviving cats which would seem to imply central emetic rather than local emetic action. Some cats did vomit during the six and one-half hour immobilization period as indicated in Table III. Of interest is the fact that vomiting was not induced to the same extent by all cardiotonic substances. Fresh tinctures of *Digitalis lanata, sibirica,* and *orientalis* did not induce vomiting, whereas fresh tinctures of *D. purpurea* and *D. lutea* as well as year-old tinctures of *D. lanata* caused an appreciable amount of vomiting. At the highest dose of *D. purpurea* used there was 100% vomiting. Therefore, in order to establish an oral LD_{50} estimate it was necessary to use dosage levels below this ceiling. Table III shows that of the purified materials studied digitoxin was much less emetic than amorphous gitalin.

DISCUSSION

Among other workers who have studied absorption of cardiac glycosides in animals, Elmqvist, Karnell, and Rydin (7) made comparisons between *Digitalis purpurea* and two samples of *D. lanata* in several species of animals. In cats they gave by means of stomach tube relatively high doses of extracts of *purpurea* and *lanata* each day and determined the number of days required to kill the cats. The mean number of survival days in the *lanata* group was 3.8 and in the *purpurea* group 9.1 days. These estimates of relative potency are almost identical with

TABLE III.—INCIDENCE OF VOMITING IN IMMOBILIZED CATS GIVEN CARDIOTONIC DRUGS BY STOMACH TUBE (SIX AND ONE-HALF-HOUR OBSERVATION PERIOD)

Drug	No. Cats Vomited/ No. Cats Treated	Lowest Dose Causing Emesis, mg./Kg.	Remarks
Digitoxin	1/55	0.43	.....
Gitalin	7/52	1.3	3 of 8 cats vomited at 2.0 mg./Kg.
D. purpurea Tr.	8/48	250.0	4 of 4 cats vomited at 4.2 cc./Kg.
D. lutea Tr.	6/36	150.0	5 of 15 cats vomited at 2.5 cc./Kg.
D. lanata fresh Tr.	0/32	....	.....
Year-old Tr.	4/40	75.0	Vomiting at 3 of 4 dose levels
D. sibirica			
Fresh Tr.	0/30	....	.....
Year-old Tr.	1/21	133.0	.....
D. orientalis	0/34	....	.....

my LD_{50} estimates of 1.03 and 2.44 cc./Kg. for *lanata* and *purpurea* tinctures respectively. The single dose method reported here was easier to control and provided a means of estimating absorption by determining the ratio between average i. v. lethal dose and oral LD_{50}. Thus this method was used in the present study.

Biological assay methods have in general utilized the intravenous route of administering cardiotonic drugs, whereas therapeutic use of these drugs requires absorption from the intestinal tract. Many years ago *D. purpurea* was found to react differently in heterologous species. Within the same species, however these data indicate that the amount of drug required to kill depends upon route of administration. This means that intravenous testing introduces a bias for orally administered drugs. Cardiotonic mixtures containing digitoxin predominantly can be expected to give reliable estimates of potency for oral use, but mixtures containing large quantities of gitoxin and other poorly absorbed materials tested intravenously can be expected to give biased estimates of potency when the drugs are used orally. For example, when absorption was taken into consideration *D. orientalis* Tr. was not the most potent tincture as indicated by intravenous testing (Table II) but was less potent than *D. lanata* and *D. sibirica* tinctures.

The method for determining potency outlined in this report perhaps is too cumbersome to be used as a biological assay procedure but such a method would test the potency, not of all cardiotonic materials present, but of the absorbable cardiotonic materials present in a crude plant glycoside mixture.

The symptoms of nausea and vomiting have been used by the clinician to indicate overdosage of *Digitalis purpurea* for many years. The data reported in this study, however, indicate that vomiting is not a reliable criterion of impending toxicity in all cardiotonic agents. Digitoxin was found to be much less emetic than gitalin, and tinctures of *D. purpurea* and *lutea* were equally effective in inducing emesis, whereas tinctures of *D. sibirica* and *orientalis* did not induce emesis. Aging seemed to increase the emetic action in *D. lanata* tincture concomitant with loss of potency intravenously as well as orally. These differences present the possibility that vomiting may be induced by some chemical substance which is a degradation product or it may be due to an inactive cardiotonic ingredient in the mixture.

Gold, *et al.* (8), focused attention on emetic action and mentioned the clinical implications of differences in emetic action in different glycosides. Since digitoxin is being used extensively in place of digitalis, relative differences in emetic action of these two drugs should be considered clinically. Gitalin appears to be more like *Digitalis purpurea* in respect to vomiting than does digitoxin.

CONCLUSIONS

1. LD_{50} estimates in unanesthetized cats were obtained by determining per cent mortality after stomach tube administration of cardiotonic drugs. The drugs tested were digitoxin, gitalin, gitoxin, and tinctures of *Digitalis lanata*, *lutea*, *sibirica*, *orientalis*, and *purpurea*. Differences in slope of the various dosage-mortality curves were not statistically significant so that the drugs studied differed quantitatively but not qualitatively.

2. Orally, digitoxin was absorbed completely; gitalin, 85%; and gitoxin not at all. About one fourth of the intravenously active material was absorbed from *D. purpurea* given orally. The other tinctures studied were very similar to *purpurea* in absorption except for *orientalis* in which 13% of the active materials were absorbed.

3. Cardiotonic drugs differ in their emetic action. For example, digitoxin induces little and gitalin relatively more emesis. Tinctures of *D. purpurea* and *lutea* induced more emesis than the others. On standing one year tincture of *D. lanata* lost in cardiotonic activity but gained in emetic action. In cats, therefore, emesis is not a uniformly reliable criterion indicating overdosage for all cardiotonic drugs.

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A Study of Anti-infectives in Dermatologic Emulsions*

II. Stability in Simple and Compound Emulsions

By KRISHNA C. VARMA†, NATHAN A. HALL, and L. WAIT RISING

The seven anti-infective agents previously studied were incorporated in four simple emulsions and four compound emulsions which contained fats, waxes, and nonionic emulsifiers. The stability of the anti-infective in each formula at room temperature over a four-week period was followed by the agar-cup-plate method. Penicillin, chlortetracycline, and bacitracin were more stable in simple emulsions. Chloramphenicol, streptomycin, Actamer®, and hexachlorophene were quite stable under the experimental conditions.

THE EFFICACY of any medicated dermatologic preparation should obviously depend upon the stability of the active ingredient in the complex system. The following study was undertaken preliminary to the exploration of some of the factors which might adversely affect the action of anti-infectives in emulsion systems on short-term storage.

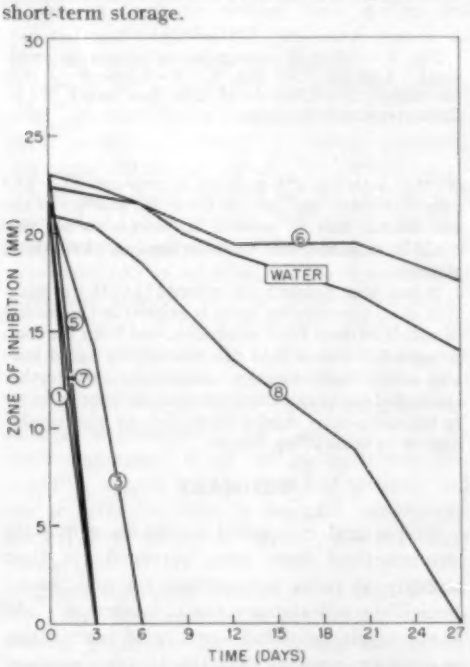

Fig. 1.—Effect of storage on penicillin emulsions. 1.—Atlas o/w; 2.—Atlas w/o (no zone); 3.—Cholesterol o/w; 4.—Cholesterol w/o (no zone); 5.—Span® 40; 6.—Arlacel® 60; 7.—Glyceryl monostearate, S.E.; 8.—Tween® 80.

* Received August 27, 1954, from the College of Pharmacy, University of Washington, Seattle.
Presented to the Scientific Section, A. PH. A., Boston meeting, August 1954.
Based on a dissertation submitted by K. C. Varma to the Graduate School, University of Washington, in partial fulfillment of the requirements for the Ph.D. degree.
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EXPERIMENTAL

Seven anti-infective agents were incorporated in four simple emulsions and four compound emulsions. The anti-infectives were penicillin G potassium, chlortetracycline hydrochloride, chloramphenicol, bacitracin, streptomycin sulfate, Actamer® [2,2′-dithio-bis-(4,6-dichlorophenol)] and hexachlorophene [2,2′-methylene-bis-(3,4,6-trichlorophenol)]. For comparison aqueous solutions of the antibiotics were run concurrently. The stability of the anti-infectives in each formula was followed over a four-week period at room temperature (24 ± 2°) by the agar-cup-plate method previously used (1).

The emulsion formulas employed were as follows:

1. *Atlas* o/w (2)		2. *Atlas* w/o (2)	
Stearic acid	7.0	Mineral oil	23.0
Lanolin anhydrous	0.5	Beeswax	1.0
Span® 80	0.5	Span® 60	2.0
Tween® 60	2.5	Arlacel® C	1.7
Water	89.5	Tween® 60	1.7
		Water	60.6
3. *Cholesterol* o/w (3)		4. *Cholesterol* w/o (3)	
Amerchol L 101®	10.0	Amerchol L 101	10.0
Paraffin wax	1.5	Lanolin	3.0
Petrolatum	3.0	Beeswax	2.0
Stearic acid	5.0	Mineral oil	30.5
Triethanolamine	1.0	Petrolatum	6.5
Water	100.0	Borax	0.5
		Water	46.5
5. Peanut oil	50.0	6. Peanut oil	50.0
Water	50.0	Water	50.0
Span® 40	1.5	Arlacel® 60	1.5
7. Peanut oil	50.0	8. Peanut oil	50.0
Water	50.0	Water	50.0
Glyceryl monostearate, self-emulsifying	1.5	Tween® 80	10.0

Chloramphenicol, hexachlorophene, and Actamer® showed no significant loss of activity during the test period, however, the other agents were variously affected during storage in the several systems (Fig. 1–4).

Under the conditions of the test the chlorinated phenols, Actamer® and hexachlorophene, were most active in the cholesterol emulsions. Actamer® showed almost twice as large a zone of inhibition in the cholesterol w/o emulsion as in the simple emulsions. Hexachlorophene exhibited a similar

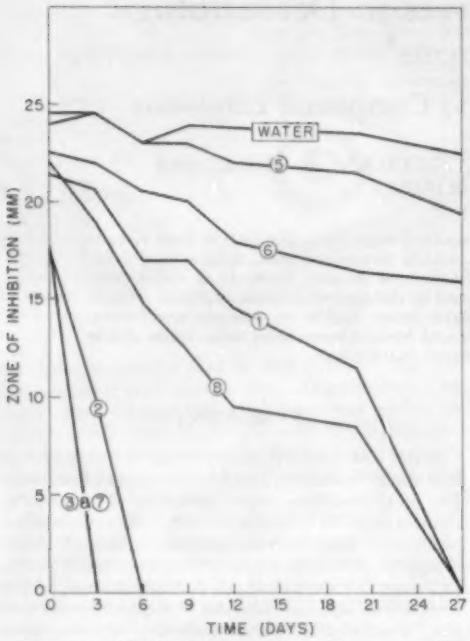

Fig. 2.—Effect of storage on chlortetracycline emulsions. Legend.—*See* Fig. 1. 4.—Cholesterol w/o (no zone).

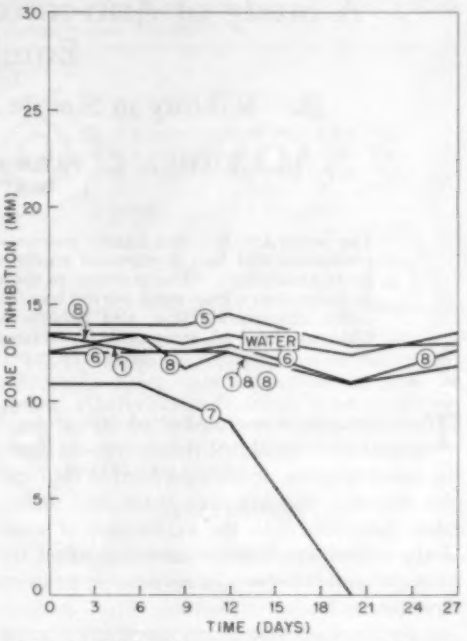

Fig. 4.—Effect of storage on streptomycin emulsions. Legend.—*See* Fig. 1. 2.—Cholesterol w/o (no zone); 3.—Cholesterol o/w (no zone); 4.—Cholesterol w/o (no zone).

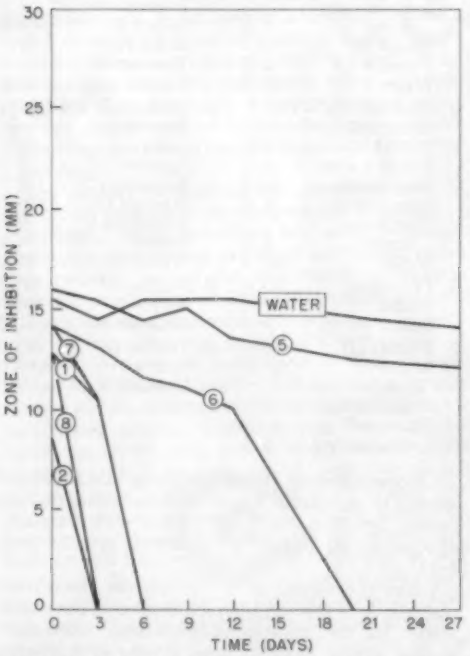

Fig. 3.—Effect of storage on bacitracin emulsions. Legend.—*See* Fig. 1. 3.—Cholesterol o/w (no zone); 4.—Cholesterol w/o (no zone).

order of activity, although not as pronounced. The simple systems appeared to favor the activity of the antibiotics, and in several instances no activity could be demonstrated with the compound emulsion systems.

It has been pointed out before (1) that the selection of an emulsifying agent is critical in the release of anti-infectives from emulsions, and from the data presented it seems that the emulsifying agent may also affect their stability. However, only further controlled study can show whether the poor stability in the compound emulsions is due to the emulsifiers or to some other factors.

SUMMARY

Simple and compound emulsions containing anti-infectives have been surveyed for their stability at room temperature for four weeks. Penicillin, chlortetracycline, bacitracin, and streptomycin exhibited more rapid inactivation in emulsion systems than the aqueous solution; but chloramphenicol, hexachlorophene, and Actamer® were quite stable under the conditions of the test.

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Determining the Efficacy of Preservatives in Pharmaceutical Products*

By E. J. RDZOK, W. E. GRUNDY, F. J. KIRCHMEYER, and J. C. SYLVESTER

A standardized method of testing the efficacy of preservatives in pharmaceutical products is presented. The test consists of the addition of a predetermined number of microorganisms of different species to the product, incubation of the contaminated test material, and sampling at fixed time intervals to determine whether growth of the organisms occurs or the preservative inhibits or kills the contaminants. The method is adaptable to multiple dose parenteral products, ophthalmic preparations, and oral or topical preparations. Where a preservative problem is involved, experience has shown that each new formulation should be examined.

THE PROBLEM of resistance to microbial growth in multiple-dose pharmaceutical products has become increasingly important to the pharmaceutical industry during recent years. Although publications of studies in this area are not extensive, a great deal of research in various laboratories has been carried out on this problem. Lord and Husa (1) and Aalto, Firman, and Rigler (2) have reported on their studies and pointed out some of the problems related to the selection of a preservative agent.

Satisfactory preservative properties in multiple dose pharmaceuticals such as ointments, parenteral products, tablets, ophthalmic products, are important for two reasons, i. e., microbiological stability of the product and the protection of the patient. The problem of selecting a preservative for a particular product involves many considerations in addition to the specific antimicrobial activity of the preservative agent. Compatibility of the preservative chemical and the active drug, the *p*H of the preparation, the presence of materials antagonistic to the preservative and many other factors must be considered as pointed out by Hartshorn (3). An important aspect of the problem of testing preservative agents has been the test methods and the criteria of what constitutes satisfactory preservative properties in a pharmaceutical product.

In our laboratory, studies in the field of preservatives have resulted in the development of several procedures for determining satisfactory preservative action and certain criteria with respect to the finished pharmaceutical have been established. It was felt that a report on the methods employed and the criteria used in determining the efficacy of preservative action would be of general interest to those working in this field. The purpose of this paper is to report the results of the studies in our laboratories on the general problem of preservative action in multiple dose pharmaceutical products.

* Received April 29, 1955, from the Research Division, Abbott Laboratories, North Chicago, Ill.

EXPERIMENTAL PROCEDURE

Principle of the Test.—The test procedures are designed to determine whether organisms when added to a pharmaceutical preparation will grow, remain static, or die. Test organisms are added in definite known amounts, and at predetermined intervals samples are taken to determine the effects of the preservative. Three modifications of the basic procedure are employed. The specific test used depends upon whether the particular product being studied is a parenteral product, an ophthalmic preparation, or a product where the problem is one of microbiological spoilage.

The Preservative Test Applied to Parenteral Products.—If the vial of the parenteral product holds 10 ml. or more of material, the test can be carried out in the vial itself. When the vials contain less than this amount, it is advisable to transfer the material to sterile screw-capped tubes in the amount of 10 ml. This amount is necessary when it is desired to prolong the test for twenty-one or twenty-eight days. The preservative test requires the use of seven test organisms. One-tenth milliliter of each diluted spore or cell suspension is added to a test vial, one organism per vial, to give a cell count of approximately 10,000 to 50,000 cells per ml. of the test material. For example, *E. coli* which gives 60,000,000 cells per ml. when standardized to 80 per cent light transmission can be diluted 1:20 to yield 3,000,000 cells per ml. One-tenth milliliter of this dilution added to 10 ml. of test material yields 30,000 cells per ml. The inoculum volume is set at one per cent to minimize the volume change. The contaminated vials are incubated at 30° for the duration of the test. The vials are sampled after one, three, and seven days and wherever indicated at weekly intervals up to a month. Plate counts are done in duplicate with dilutions of 1:10, 1:100, and 1:1000 using B-1 agar. The results of the plate count will show whether the preservative is bacteriostatic, bactericidal, or ineffective.

The Preservative Test Applied to Ophthalmic Preparations.—These products are handled in the same manner as injectable materials. However, since in the use of these products the containers are repeatedly opened to the air and portions removed with droppers, it is necessary to give the product a

more vigorous testing. In addition to the standard seven-organism test, three or more *Pseudomonas aeruginosa* strains are included since this organism is the most troublesome in this type of product (4).

The Spoilage Test.—This test is applied to products for oral or topical use other than ophthalmic products. Contamination is accomplished with dried garden soil which in addition to its normal flora also contains spores of nine added molds. One milligram of soil added to each Gm. or ml. of test material is the routine method of contamination. The contaminated product is incubated at 30° for one month or longer and is examined once a week by means of the plate count. Sabouraud's agar[1] is used for the mold and yeast count while B-1 agar is used for the bacterial count.

Syrups with a high sugar content raise another problem. In addition to using the dried soil technique another test is run which incorporates an osmophilic yeast. Contamination is accomplished with a seventy-two hour broth culture (Sabouraud's liquid medium[1]) of an osmophilic yeast strain OY-1. A one per cent inoculum of undiluted culture is added to the test material. The contaminated product is incubated at 30° for two months or longer and is examined once a week by means of plate counts. The malt extract-yeast extract medium of Wickerham (5) is used for the osmophilic yeast counts.

Preparation of Test Cultures for Preservative Testing.—The seven test organisms employed include an aerobic spore forming rod, *Bacillus cereus* A.T.C.C. 7064; a mold, *Aspergillus niger* A.T.C.C. 6277; a yeast, *Candida albicans* A.T.C.C. 10231; two cocci, *Streptococcus faecalis* A.T.C.C. 10541 and *Micrococcus pyogenes* var. *aureus* A.T.C.C. 6538 P; and two Gram-negative rods, *Escherichia coli* A.T.C.C. 6880 and *Pseudomonas aeruginosa* A.T.C.C. 10145. The cultures *B. cereus* and *A. niger* are employed as spore suspensions while the remaining five are used as standardized suspensions of vegetative cells.

The spore suspension of *Bacillus cereus* is prepared by inoculating a 500-ml. Erlenmeyer flask containing 100 ml. of Tryptose Phosphate broth[2] with 2 ml. of a twenty-four hour broth culture. The flask is incubated on a rotary shaking machine having an offset of 2¼ inches and speed of 240–260 r. p. m. for four days at 28°. The cells are centrifuged, washed twice, resuspended in sterile water, and placed in a sterile bottle containing glass beads (approx. 75 ml. per bottle). The suspension is pasteurized for twenty minutes at 80° and stored in the cold room. The spore count of the suspension is established by several plate counts. The desired number of spores, 10,000 to 50,000 per ml., in the test material is obtained by appropriate dilution of the stock. This spore suspension can be used for several months without appreciable loss of cell count.

Aspergillus niger is first grown on slants of Sabouraud's agar at 28° for three to four days. It is then transferred to a flask of wheat bran medium and incubated for six days at 28°. On the day of harvest 100 ml. of sterile water is added to the flask, the flask is placed on the rotary shaking machine for one-half hour to break up the clumps and to wet the spores. This suspension is filtered through sterile glass wool, to separate the spores from the mycelium and bran. The spore suspension is stored in the cold room and may be used for a period of several months.

Fresh cell suspensions are prepared for the other five test organisms. Stock cultures are kept on tryptose phosphate agar slants. The cultures are transferred to tryptose phosphate broth, incubated at 37° for twenty-four hours, and transferred back to slants for the same period of incubation. The cells on these slants are washed off with water, and used to prepare the standardized suspensions with the aid of a Lumetron colorimeter, Model 402E. The following table gives the cultures, per cent light transmission as compared to distilled water at 100 per cent, and corresponding plate counts.

TABLE I.—PLATE COUNTS OBTAINED WITH STANDARDIZED CELL SUSPENSIONS

Culture	Per cent Light Transmission	Plate Count, million/ml.
E. coli	80	50–60
Ps. aeruginosa	80	40–50
M. pyogenes var. *aureus*	90	25–35
Strep. faecalis	90	15–20
C. albicans	80	1.5–2.0

Preparation of the Soil Stock and the Osmophilic Yeast Culture for the Spoilage Test.—Garden soil, in addition to its normal flora, is supplemented by the addition of the spores of nine molds. The molds are: *Monilia sitophila*, a *Mucor* species, a *Hormodendron* species, *Chaetomium globosum*, an *Alternaria* species, a *Cephalosporium* species, *Aspergillus niger*, a *Paecilomyces* species, and a *Penicillium* species. Individual slants of the molds are grown and incubated until fully sporulated. A spore suspension of each is added to sterile soil in tubes and allowed to dry. All nine soil spore suspensions are pooled and blended. One part of mold soil spore suspension is added to nine parts of dried garden soil. This material is used in the spoilage test.

The osmophilic yeast culture OY-1 was isolated in this laboratory from an experimental product containing no preservative. This yeast culture is kept in stock on malt extract-yeast extract agar slants. A transfer is made from a slant to a tube of Sabouraud's liquid medium and incubated at 30° for seventy-two hours. This culture is then used to contaminate the syrup on test. A one per cent inoculum yields approximately 50,000 yeast cells per ml.

Media.—*Sabouraud's liquid medium* (15 Gm./L. of agar for solid medium).

Tryptose phosphate broth (15 Gm./L. of agar for solid medium).

B-1 Agar.—Tryptone—3 Gm.; beef extract, 3 Gm.; glucose, 1 Gm.; yeast extract, 1 Gm.; agar, 15 Gm.; tap water, 1 L.; *p*H 7.0–7.2.

Wheat Bran Medium.—Sabouraud's liquid medium 70 ml.; wheat bran, 100 Gm. Mix well.

Malt Extract-Yeast Extract Medium.—Malt extract, 3.0 Gm.; yeast extract, 3.0 Gm.; peptone, 5 Gm.; glucose (commercial grade), 20.0 Gm.; water, 1 L.; agar, 30 Gm. Mix all ingredients except the sugar in the water, melt agar, add sugar, dissolve, do not adjust *p*H, autoclave at 12–15 pounds pressure for fifteen to twenty minutes.

[1] Baltimore Biological Laboratory Inc., Baltimore 18, Md.
[2] Difco Laboratories Inc., Detroit 1, Mich.

RESULTS AND DISCUSSION

Data obtained with experimental pharmaceutical formulations are presented to illustrate how the test methods determine the effectiveness or failure of a preservative. In order for a preservative to be considered satisfactory it must exhibit a bacteriostatic or bactericidal effect upon the spore-forming organisms and a bactericidal effect upon the non-spore-forming organisms.

The Preservative Test Applied to Parenteral Products.—The product tested was sterile estrone aqueous suspension 2 mg./ml., 20,000 International Units, 10-ml. vial, containing methylparaben 0.18% and propylparaben 0.02% as preservatives. The results are presented in Table II.

TABLE II.—RESULTS OF THE PRESERVATIVE TEST APPLIED TO A MULTIPLE DOSE PARENTERAL PRODUCT

Estrone Aq. Susp. with Methylparaben 0.18% and Propylparaben 0.02%

Culture	Plate Count 0 days	1 day	3 days	7 days
B. cereus spores	22,000	21,500	17,000	18,500
E. coli	38,000	<10	<10	<10
Ps. aeruginosa	32,000	<10	8,100	63,500
Strep. faecalis	26,000	29,500	34,000	16,500
M. pyogenes var. *aureus*	20,000	<10	<10	<10
C. albicans	21,000	2,450	<10	<10
A. niger spores	34,000	320	50	<10

The preservative action of the product was considered unsatisfactory, because of the growth of *Ps. aeruginosa* and a lack of bactericidal effect on *Strep. faecalis*.

A different preservative, 0.9% benzyl alcohol, was added to this product in place of the parabens. The results are given in Table III.

TABLE III.—RESULTS OF THE PRESERVATIVE TEST APPLIED TO A MULTIPLE DOSE PARENTERAL PRODUCT

Estrone Aq. Susp. with 0.9% Benzyl Alcohol

Culture	Plate Count 0 days	1 day	3 days	7 days
B. cereus spores	32,000	30,500	29,000	27,000
E. coli	57,000	<10	<10	<10
Ps. aeruginosa	50,000	<10	<10	<10
Strep. faecalis	31,000	<10	<10	<10
M. pyogenes var. *aureus*	34,000	<10	<10	<10
C. albicans	28,000	<10	<10	<10
A. niger spores	47,000	12,000	10	<10

The data in Table III indicate that 0.9% benzyl alcohol has satisfactory preservative properties for this product.

To illustrate further the effectiveness of a preservative in an injectable material, data taken from another pharmaceutical are presented. The product tested was succinylcholine chloride 20 mg./ml. containing methylparaben 0.18% and propylparaben 0.02% as preservatives. The results are given in Table IV.

TABLE IV.—RESULTS OF THE PRESERVATIVE TEST APPLIED TO A MULTIPLE DOSE PARENTERAL PRODUCT

Succinylcholine Chloride with Methylparaben 0.18% and Propylparaben 0.02%

Culture	Plate Count 0 days	1 day	3 days	7 days
B. cereus spores	32,000	19,500	13,500	11,000
E. coli	57,000	25	<10	<10
Ps. aeruginosa	53,000	<10	<10	<10
Strep. faecalis	31,000	<10	<10	<10
M. pyogenes var. *aureus*	34,000	<10	<10	<10
C. albicans	28,000	<10	<10	<10
A. niger spores	47,000	40	<10	<10

These data demonstrate that the two parabens are satisfactory for this product.

The need to examine each product under development is emphasized by the data from the estrone preparation. The mixture of methyl and propyl parabens was an excellent preservative with succinylcholine chloride but was unsatisfactory with estrone aqueous suspension even though the *p*H was adjusted to 4.4–5.0 which is the most effective range for the parabens. These results were not predictable from the previous information available and came to light only after the completion of the preservative tests.

The Preservative Test Applied to Ophthalmic Preparations.—Data taken from the development of an ophthalmic product are presented to illustrate the necessity for the proper concentration of preservative. The product tested is known as Lacril. One sample contained benzethonium chloride in the concentration of 1:50,000, whereas the other sample contained the preservative in the concentration of 1:5,000. The results are given in Tables V and VI. In Table V it can be seen that benzethonium chloride in a concentration of 1:50,000 is unsatisfactory against the *Pseudomonas* strains. In Table VI the results indicate that the preservative in a concentration of 1:5,000 in the product, Lacril, affords excellent preservative properties.

The Spoilage Test.—Data from product development are presented here on three preparations of selenium sulfide jelly. Samples A and B contained no preservative whereas sample C contained phenylethyl alcohol 0.5% as preservative. The results are given in Table VII.

Samples A and B were both spoiled as evidenced by their odor and change of consistency after the first week of incubation. The preservative properties of Sample C were satisfactory.

The spoilage test as applied to syrups is illustrated by data from three preparations of Vita-King, a vitamin preparation for animals. Sample A contained 0.2% benzoic acid and 0.06% methylparaben. Sample B contained 0.15% benzoic acid and 0.045% methylparaben. Sample C contained 0.12% benzoic acid and 0.036% methylparaben. The spoilage yeast OY-1 was added to each material. The results are given in Table VIII.

The data indicate that the preservatives in samples B and C are not of sufficient concentration to eliminate quickly the yeast. In contrast the preservatives in sample A readily killed the yeast and this formulation is considered satisfactory.

TABLE V.—RESULTS OF THE PRESERVATIVE TEST APPLIED TO AN OPHTHALMIC PREPARATION

Lacril with Benzethonium Chloride 1:50,000				
	Plate Count			
Culture	0 days	1 day	3 days	7 days
Ps. aeruginosa	62,500	67,500	116,000	31,000
Ps. aeruginosa No. 1	62,500	35,000	>300,000	>300,000
Ps. aeruginosa No. 4	35,000	113,000	>300,000	>300,000
Ps. aeruginosa No. 10	63,000	47,000	12,000	>300,000

TABLE VI.—RESULTS OF THE PRESERVATIVE TEST APPLIED TO AN OPHTHALMIC PREPARATION

Lacril with Benzethonium Chloride 1:5,000				
	Plate Count			
Culture	0 days	1 day	3 days	7 days
Ps. aeruginosa	62,500	<10	<10	<10
Ps. aeruginosa No. 1	62,500	<10	<10	<10
Ps. aeruginosa No. 4	35,000	<10	<10	<10
Ps. aeruginosa No. 10	63,000	<10	<10	<10
B. cereus spores	35,600	11,200	6,500	3,000
E. coli	44,000	<10	<10	<10
Strep. faecalis	52,000	<10	<10	<10
M. pyogenes var. *aureus*	41,000	<10	<10	<10
C. albicans	23,000	<10	<10	<10
A. niger spores	73,000	<10	<10	<10

TABLE VII.—RESULTS OF THE SPOILAGE TEST ON SELENIUM SULFIDE JELLY

	Plate Count		
Time	Sample A	Sample B	Sample C
1 week	53,000	>300,000	5,000
2 weeks	>300,000	>300,000	10,000
3 weeks	>300,000	>300,000	4,700
4 weeks	>300,000	>300,000	6,400

TABLE VIII.—RESULTS OF THE SPOILAGE TEST ON THREE PREPARATIONS OF VITA-KING

	Yeast Count per Gram		
Incubation Time at 30°	Sample A 0.2% Benzoic Acid 0.06% Methyl-paraben	Sample B 0.15% Benzoic Acid 0.045% Methyl-paraben	Sample C 0.12% Benzoic Acid 0.036% Methyl-paraben
0 days	55,400	55,400	55,400
1 week	10	860	8,100
2 weeks	None detected	265	6,900
3 weeks	None detected	160	5,150
4 weeks	None detected	140	1,825
5 weeks	None detected	55	500
6 weeks	None detected	20	305
7 weeks	None detected	10	150
8 weeks	None detected	None detected	75

SUMMARY

1. A standardized method of testing the efficacy of preservatives in pharmaceutical products is presented.

2. The method is adaptable to multiple dose parenteral products, ophthalmic preparations, and oral or topical preparations.

3. A few typical results from product development are presented to illustrate the application of the test in a pharmaceutical laboratory.

4. Where a preservative problem is involved, experience has shown that each new formulation should be examined.

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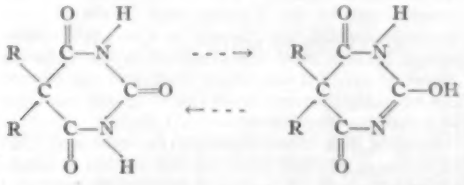
Tautomerism in Solid Secobarbital Sodium*

By A. JOHN SPEZIALE, WILLIAM E. KOERNER, FERDINAND C. MEYER, and RALPH H. MUNCH

Isolation of sodium 5-allyl-5-(1-methylbutyl) barbiturate from anhydrous butanol or aqueous ethanol gave rise to two products which differed in some physical properties. The material from the butanol procedure appeared to contain 30.7 per cent impurities whereas that from aqueous ethanol contained 9 per cent when both were subjected to the minimum solubility purity test. When the butanol material was reworked via the ethanol method, a purity test of the sample revealed the presence of only 3.1 per cent impurities. A consideration of the differences in the two procedures indicated that the presence or absence of water during the recovery of the solid secobarbital sodium influenced the amido-imidol equilibrium in the recovered solid. This hypothesis was confirmed by infrared absorption spectra of mulls of the solid materials. The anhydrous butanol material gave a product whose amido-imidol equilibrium was displaced more towards the imidol form than the aqueous ethanol material. The displacement of tautomeric equilibrium of properly substituted sodium barbiturates isolated from anhydrous alkanol media may be general.

PREVIOUS WORKERS have examined the ultraviolet absorption spectra of aqueous solutions of barbiturates at varying *p*H (1) and have interpreted them as evidence for tautomerism. In 5,5-disubstituted barbituric acids, tautomerization can involve only the —CO—NH—CO—NH—CO— moiety. Stuckey (1d, e) has interpreted the amido-imidol tautomerism as involving only one hydrogen atom and the number 2 carbonyl group.



The pharmacological action of 5,5-disubstituted barbituric acids has been reported to be related to this tautomerization (1c) and to the ionization constant of the acid (2). Methylation of disubstituted barbituric acid with diazomethane (3, 4) or methyl sulfate (1e), however, yields the N-methyl and not the O-methyl derivative. From a study of the reaction of diazomethane with barbituric acids and similarly constituted compounds, Arndt (4) has concluded that the two CONH groups do not enolize (although the NH group is acidic) but participate to give rise to the resonance hydrid through aromatization.

Two forms of the neutral cobalt salt of 5-phenyl-5-ethyl barbituric acid have been reported (5). Although these compounds differ in color and chemical properties, the differences are not sufficiently pronounced to postulate definitely the existence of two tautomeric salts.

We wish to report our results on the variation of the ratio of tautomers in solid sodium 5-allyl-5-(1-methylbutyl) barbiturate (secobarbital sodium) isolated from aqueous and non-aqueous media.

5-Allyl-5-(1-methylbutyl) barbituric acid was prepared by the allylation (6) of 5-(1-methylbutyl) barbituric acid. The crude barbituric acid was converted to its sodium salt by solution in water under controlled *p*H. Two procedures were used for the isolation of the dry sodium salt. The first involved the addition of ethanol to the aqueous solution and evaporation to dryness to recover all material originally in solution. In the second method butanol-1 was added to the aqueous solution, and the water removed by azeotropic distillation. The clear, anhydrous solution was cooled to crystallize the sodium salt which was filtered, washed with anhydrous butanol, and dried. The latter procedure allows the removal of impurities via the butanol-1 mother liquor and was expected to yield a purer product than the ethanol procedure.

The purity of the products from the two procedures was determined by a minimum solu-

* Received April 1, 1955, from Monsanto Chemical Company, Research Department, Organic Chemicals Division, St. Louis 4, Missouri.

We would like to express our appreciation to Mr. O. E. Kinast who obtained the infrared absorption spectra of the sample, and to Mr. William Hewitt and Mr. Robert Corning for numerous analyses.

bility purity test using anhydrous 2-methylbutanol-2 as the solvent.

The initial results from the minimum solubility purity tests seemed to be a direct contradiction of our expectations. The material from the ethanol recovery method appeared to contain about 9 per cent impurities whereas a sample from the same allylation batch which was isolated by the butanol-1 procedure appeared to contain 30.7 per cent impurities. The impurities in the material recovered from ethanol might be the sodium salt of 5-allyl-5-(1-ethylpropyl) barbituric acid (6b). Consideration of the differences in the two procedures led us to suspect that the presence or absence of water during the recovery of the solid secobarbital sodium might influence the amido-imidol equilibrium in the recovered solid. The nature of the minimum solubility purity test is such that any material present in the other isomeric form would be considered as impurity. Furthermore the 2-methylbutanol-2 used for the purity test was nearly anhydrous and hence would not be considered a good solvent for the attainment of tautomeric equilibrium approximating that existing in aqueous solution. To test this hypothesis a sample of the material recovered from the butanol-1 process was dissolved in water and recovered by the ethanol process. This mild treatment offered little, if any, opportunity for the introduction or removal of impurities. A purity test on this sample revealed the presence of only 3.1 per cent impurities.

Solubility purity tests on mixtures of ethanol processed and butanol-1 processed secobarbital sodium resulted in apparent impurity contents intermediate between the values which had previously been obtained for the samples from which the mixtures were prepared. This indicates that different crystal forms could not have been responsible for the differences between the ethanol processed and butanol-1 processed samples.

To check further the validity of our hypothesis, the infrared absorption spectra of mulls of the butanol-1 procedure material before and after the reworking by the ethanol procedure were obtained. The sample recovered by the butanol-1 procedure showed stronger associated hydroxyl group absorption in the 2.9 micron region. Thus it appears that a larger fraction of the material recovered from the butanol-1 procedure is in the imidol form.

There were no essential differences in the ultraviolet absorption spectra of aqueous solutions of material isolated by either of the above two methods, the butanol material reworked with ethanol, or a commerical sample of secobarbital sodium. The different tautomeric equilibrium in the butanol material would not be evident in the ultraviolet analysis, since the material, whichever method of isolation was used, on dissolution in water would equilibrate to the normal tautomeric ratio for an aqueous solution of the salt.

It is evident that isolation of secobarbital sodium from anhydrous butanol-1 gives a product whose amido-imidol equilibrium is displaced more towards the imidol form than when an aqueous solvent is employed in its isolation.

The displacement of tautomeric equilibrium of properly substituted sodium barbiturates effected by isolation from anhydrous alkanol media may be general.

EXPERIMENTAL

Sodium 5-Allyl-5(1-methylbutyl) Barbiturate (Secobarbital Sodium).—A modification of the methods of Shonle (6a) and Tabern (6b) was employed.

In one allylation experiment half of the material was recovered from butanol-1 and the other half from ethanol.

The dried material isolated by the butanol-1 method was later dissolved in water and isolated according to the ethanol method.

Solubility Purity Test.—The basic principles of the minimum solubility purity test have been discussed recently by Tarpley and Yudis (7). 2-Methylbutanol-2 was chosen as the equilibration solvent because secobarbital sodium is soluble to the extent of only 2.2 weight per cent, and the solvent can be easily removed from the saturated solutions in a vacuum evaporator.

Equilibration time experiments indicated that 45 hours of efficient agitation was needed to establish equilibrium. A portion of the filtered, saturated solution was titrated with Karl Fisher reagent to determine the water content after equilibration. The solubility of secobarbital sodium is increased 0.00028 gram per gram of 2-methylbutanol-2 for each increase of 0.01% in the water content of the 2-methylbutanol-2.

The observed solubilities for the different solid-to-solvent mixtures prepared from any given sample were corrected to the same water content. The experimental data which we obtained are shown in Table I.

The corrected concentrations of the different saturated solutions, expressed as grams of solid dissolved per gram of solvent, were plotted against the solid-to-solvent ratio used, expressed as grams of sample per gram of solvent. The data for each sample plotted as a straight line, and the apparent impurity contents of the samples were calculated from the slopes of the lines.

The infrared absorption spectra were measured as 40% Nujol mulls on a Model 21 Perkin-Elmer spectrophotometer.

TABLE I.—MINIMUM SOLUBILITY PURITY TEST DATA

	Solid-to-Solvent Ratios, Gm./Gm.	Concentrations of Saturated Solutions—Corrected to Same H_2O Content, Gm./Gm.
Recovered from butanol-1	0.0412	0.0346
	0.0852	0.0480
	0.122	0.0592
Recovered from butanol-1, dissolved in water, and recovered by ethanol procedure	0.0382	0.0256
	0.0880	0.0271
	0.145	0.0289

SUMMARY

1. Sodium-5-allyl-5-(1-methylbutyl) barbiturate has been isolated from anhydrous butanol and aqueous ethanol.

2. Minimum solubility purity tests and infrared absorption spectra have shown these materials to differ in their composition with respect to their amido-imidol forms.

3. The two isomeric forms can be interconverted by proper choice of solvents.

4. The displacement of tautomeric equilibrium of properly substituted sodium barbiturates effected by isolation from anhydrous alkanol media may be general.

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Preliminary Studies on the Effect of Feeding Sorbic Acid Upon the Growth, Reproduction, and Cellular Metabolism of Albino Rats*

By G. E. DEMAREE, D. W. SJOGREN, B. W. McCASHLAND, and F. P. COSGROVE†

Simple observations on the amounts of food eaten daily indicate that the incorporation of 10% sorbic acid into the diet did not appreciably alter the quantity of food consumed by the test animals. Some of the test animals showed a higher growth rate and a higher ratio of liver weight to body weight than did the controls. The reproductive capacity was found to be normal. No significant differences were observed in the oxygen consumption of liver homogenates of the parent generation; the differences observed in the first generation were significant at the 95% level. The female controls of this group showed the highest rate of oxygen consumption. The rate of oxygen consumption of the liver homogenates from the males and females of this generation treated with 10% sorbic acid were almost identical.

IN A PREVIOUS PUBLICATION from this laboratory it was established that sorbic acid, CH_3—CH=CH—CH=CH—COOH,[1] is a satisfactory fungistatic agent for certain pharmaceutical preparations which are susceptible to mold growth (1). Smith and Rollin (2) also found the acid to be effective in protecting cheeses and cheese products from mold spoilage. Deuel, *et al.* (3), have reported that sorbic acid is harmless to rats and to dogs when incorporated into the diets to the extent of 5%, however, their data indicate that rats fed a diet containing 8% of the acid exhibited a slight but statistically significant increase in liver weight without signs of micropathology.

In view of these results, it was thought desirable to carry out preliminary studies on the effect of a diet containing 10% sorbic acid upon the growth and reproduction, and upon the metabolism of liver homogenates of two generations of albino rats.

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[1] Supplied by Carbide and Carbon Chemical Co., New York, N. Y.

TABLE I.—EFFECT OF 10% SORBIC ACID DIET ON GROWTH RATE OF ALBINO RATS

Days on Diet	Males: No. of Rats	Males: Control Diet Wt. in Gm. ± $S_{\bar{x}}$	Males: No. of Rats	Males: Sorbic Acid Diet Wt. in Gm. ± $S_{\bar{x}}$	Females: No. of Rats	Females: Control Diet Wt. in Gm. ± $S_{\bar{x}}$	Females: No. of Rats	Females: Sorbic Acid Diet Wt. in Gm. ± $S_{\bar{x}}$
Parent Generation								
0	5	64.6 ± 1.2	5	64.4 ± 0.8	5	63.6 ± 3.0	5	61.0 ± 1.3
30	5	235.2 ± 6.1	5	212.8 ± 9.9	5	188.8 ± 4.0	5	189.8 ± 8.2
60	5	353.8 ± 29.6	5	342.2 ± 7.5	5	242.6 ± 4.9	5	230.8 ± 10.1
120	5	428.2 ± 9.3	5	430.6 ± 7.9	5	280.4 ± 7.5	5	260.4 ± 9.7
First Generation								
40	7	311.0 ± 7.8	10	233.7 ± 8.4[a]	7	196.7 ± 6.7	9	179.1 ± 6.7
70	7	356.7 ± 8.4	10	286.2 ± 7.4[a]	7	220.9 ± 5.3	9	205.2 ± 6.8
120	5	414.6 ± 16.8	5	326.8 ± 14.0[a]	5	195.2 ± 13.8	5	219.0 ± 12.8

[a] Significant difference at 99% level.

EXPERIMENTAL

Growth Experiments.—All animals used in this study were bred from Sprague-Dawley stock and were kept in quarters which were maintained at about 25°. The animals were sexed and grouped as: male test rats, male control rats, female test rats, and female control rats. The diet of the control groups was powdered Purina Laboratory Chow and water while the test groups received such chow containing 10% of sorbic acid and water as their diet. Food and water were always available to each rat.

The weights of individual animals of the parent generation were recorded at the beginning of the study and at 30, 60, 90, and 120 days of feeding. The rats used in the second portion of the growth study were selected at random from the young born of the parent generation. The weights of these animals were taken after 40, 70, and 120 days of feeding. Results of the growth studies are shown in Table I.

Reproduction Data.—Animals of the parent generation were mated at ninety days of age and after being fed the respective diets for sixty days while the rats of the first generation were mated after ninety days of age and on these diets for a period of seventy days. All females had young within thirty days. Results are shown in Table II.

Liver Weight and Oxygen Uptake of Liver Homogenates Data.—(*a*) *Preparation of Liver Enzymes.*—The livers were removed from the rats and weighed immediately. In the case of the first generation the animals were selected at random. Results expressed as liver weight percentage body weight are recorded in Table II. The liver enzymes were then prepared according to the method of Lehninger (4) with the following modification: the final suspension was adjusted with phosphate buffer so that 1.0 ml. represented 0.40 Gm. of fresh liver. The preparations were stored at approximately 10° and used within seven hours.

(*b*) *Preparation of Reaction Mixture.*—The main compartment of a 15.0 ml. Warburg flask contained 3.0 ml. of enzyme suspension, 0.10 ml. adenylic acid solution (0.001 *M*), 0.10 ml. magnesium sulfate solution (0.005 *M*), 0.10 ml. phosphate buffer (0.0025 *M*), 0.10 ml. cytochrome C solution (1×10^{-4} *M*), and 0.10 ml. sodium succinate (0.10 *M*).

The side arm contained 0.20 ml. sulfuric acid (1 *N*), and the center well contained 0.10 ml. sodium hydroxide (1 *N*). The reaction times were 30 or 40 minutes at a temperature of 32°. A range of seven to twelve flasks were employed for each liver preparation. Results of oxygen uptake are given in Table III.

TABLE II.—EFFECT OF 10% SORBIC ACID DIET ON REPRODUCTION OF ALBINO RATS[a]

	Parent Generation: Number Mothers	Parent Generation: Mean Number of Offspring ± $S_{\bar{x}}$	First Generation: Number Mothers	First Generation: Mean Number of Offspring ± $S_{\bar{x}}$
Control Diet	5	10.0 ± 2.1	7	8.3 ± 1.8
Sorbic Acid Diet	5	9.4 ± 1.2	9	9.6 ± 1.7

[a] No significant difference found.

DISCUSSION AND SUMMARY

Throughout this study the general appearance and behavior of the animals appeared to be normal. The fact that the rats scattered some of the food made it impractical to report the exact amount of food consumed. Simple observations on the amounts of food eaten daily indicate that the incorporation of 10% sorbic acid into the diet did not appreciably alter the quantity of food consumed by the test animals. This observation is in accordance with the findings of Deuel, *et al.* (3). However, some test animals showed a higher growth rate and a higher ratio of liver weight to body weight than did the controls. Female test rats of the first generation were the only test group not showing an increase in the ratio of liver weight to body weight over the corresponding controls.

While removing the livers it was observed that male test animals of both generations possessed more depot fat than the male controls; however little or no difference was observed between females of the test and the control groups. This may be partially explained by the fact that these rats recently had produced young.

The reproductive capacity of the animals was found to be normal.

There were no significant differences observed in the oxygen consumption of liver homogenates of the parent generation, on the other hand the differences observed in the first generation were significant at the 95% level. The female control homogenates of this group showed the highest rate of oxygen consumption which might be expected because the

TABLE III.—EFFECT OF 10% SORBIC ACID DIET ON LIVER WEIGHT PERCENTAGE BODY WEIGHT AND OXYGEN UPTAKE OF LIVER HOMOGENATES OF ALBINO RATS

	Parent Generation				First Generation			
	Livers from Male Rats		Livers from Female Rats		Livers from Male Rats		Livers from Female Rats	
	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet
Oxygen Uptake[a]	160. ± 6.9	174.5 ± 4.7	186. ± 7.5	166. ± 6.0	73. ± 4.1	100. ± 8.1[b]	177. ± 3.6	99. ± 6.2[b]
% Liver wt. / Body wt.	3.33 ± 0.18	3.92 ± 0.22[c]	4.34 ± 0.50	5.59 ± 0.65[c]	3.18 ± 0.15	3.43 ± 0.59[b]	3.16 ± 0.50	3.44 ± 0.91

[a] Based on μl./40 minutes for the parent generation and μl./30 minutes for the first generation ± standard error.
[b] Significant difference at 95% level.
[c] Significant difference at 99% level.

metabolic rate of female rats is higher than that of males. The rate of oxygen uptake by the male control preparations was the lowest. It is interesting to note that the rate of oxygen consumption of the liver homogenates from both the males and females of this generation treated with 10% sorbic acid were almost identical.

Since the results of the preliminary studies show certain differences, further work is indicated to verify our results. The maximum concentration of sorbic acid in the diet, which will not produce significant changes in the functions of the liver and other organs of several animal species should be determined.

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Effect of Glutathione on the Response of *Escherichia coli* to Penicillin*

By ROBERTSON PRATT

Glutathione, in appropriate concentrations, nullifies the slight depressing effect of low concentrations of penicillin (of the order of 4–5 micrograms/ml.) against *Escherichia coli* A.T.C.C. 6522. At higher concentrations of penicillin (up to about 37 micrograms/ml.) addition of glutathione in concentrations from 0.1 to 100 times the molar concentration of the antibiotic seemed to increase the sensitivity of the organism to penicillin.

SEVERAL FACTORS suggest that study of the combined effect of glutathione and penicillin on bacteria might lead to further elucidation of the mechanism of action of the antibiotic. Involvement of —SH groups in the action of penicillin has been suggested frequently (1, 2); interference of penicillin with absorption or utilization of glutamic acid, which constitutes a moiety of the glutathione molecule, has been demonstrated (3–5); structural similarities between penicillin and glutathione have been pointed out (6, 7); and, finally, indirect evidence which suggests that penicillin interferes with the normal equilibrium between the reduced and the oxidized forms of glutathione in sensitive strains of *Micrococcus pyogenes* var. *aureus* has been reported (2, 8, 9, 10).

The present report, which is part of a larger study of different groups of organisms, is concerned with the effect of glutathione on the response of a relatively penicillin-insensitive organism (*Escherichia coli* A.T.C.C. 6522) to the antibiotic in a chemically defined medium.

EXPERIMENTAL

Methods.—*Escherichia coli* A.T.C.C. 6522 was maintained through daily transfers in Anderson's medium as modified by Cowie, *et al.* (11). In this simple medium, containing only dextrose as a carbon source and no organic nitrogen, the organisms multiply somewhat more slowly than in nutrient broth but cultures in the two media have approximately the same absorbance after eighteen hours of

* Received April 25, 1955, from the University of California School of Pharmacy, University of California Medical Center, San Francisco 22.

incubation at 37° in roller tubes rotating at a rate of 5 r. p. m. The optical density, determined after eighteen hours in tubes with inside diameter of 18 mm., is about 1.25 in synthetic medium and about 1.40 in broth. Measurements were made with a Lumetron, Model 402EF, equipped with a neutral filter.

Solutions of glutathione and of penicillin were sterilized by filtration through sintered glass. Other ingredients of the culture solutions were sterilized in an autoclave for twenty minutes at 15 pounds pressure and 120°. A potassium salt of benzyl penicillin, assaying 1,597 units per mg., was used in all experiments.

Organisms used for daily transfers were not washed, but those used for inoculating experimental tubes were spun down in a centrifuge and then were washed three times by resuspending and centrifuging in volumes of distilled water equal to the volume of the initial aliquot withdrawn from the stock culture tube. The subsequent growth rate and final absorbance were the same whether the organisms were washed with distilled water or with phosphate buffer (*p*H 6.8). Therefore, the water technic was adopted. After the third wash, the organisms were resuspended in distilled water and the absorbance of the suspension was adjusted to 47.5–50.0. One milliliter of this suspension was inoculated into 14 ml. of medium in each culture tube, giving an initial viable count of approximately 200,000,000 organisms, per ml.

Results.—The effect of different concentrations of penicillin on growth of the test organism in the synthetic medium is shown in Fig. 1. All concentrations studied (1.25×10^{-6} to 2.0×10^{-4} *M*)[1]

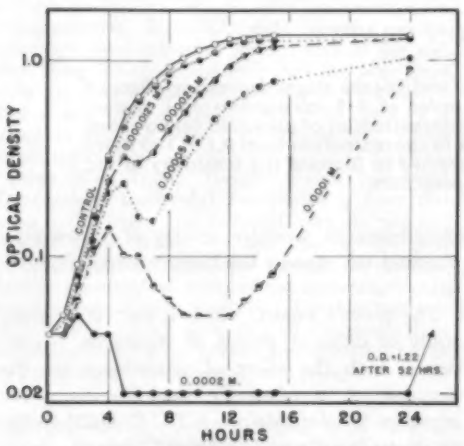


Fig. 1.—Growth of *Escherichia coli* A.T.C.C. 6522, as indicated by optical density, in a chemically defined medium with different concentrations of potassium benzyl penicillin.

caused transient depression of growth. In all but the lowest concentration of penicillin there was not only retardation of growth but also actual decrease in absorbance after an initial increase. The time of onset of depression, the amount of decrease, and the length of time before growth resumed bore approximately a linear relationship to the concentration of antibiotic. In all instances, however, ultimately the cultures resumed growth and reached the same absorbance as the control without penicillin. When growth did resume, the rate was approximately the same as in the control cultures, regardless of the concentration of penicillin to which the organisms had been exposed.

Results of the series of experiments summarized in Fig. 2 show that some thermal inactivation of penicillin occurred at the incubator temperature, but that thermal inactivation was not the major factor in the destruction (of the antibiotic) which permitted subsequent resumption of growth by the survivors. This would be expected, since the test organism produces penicillinase.

Parallel sets of tubes were prepared with media containing 5.0×10^{-5} *M* penicillin. One set of tubes was inoculated and both sets were placed in the incubator. After eight hours, at which time growth in the inoculated tubes had begun to come out of the depression caused by the penicillin, tubes

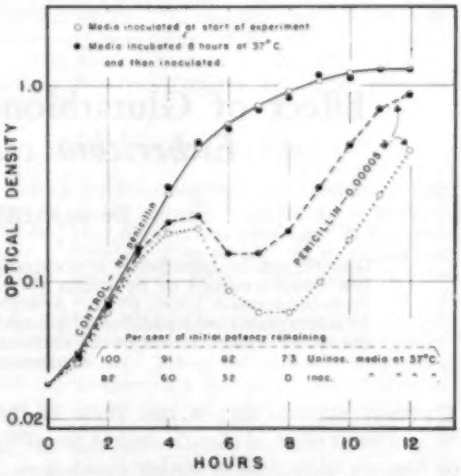


Fig. 2.—Effect of preincubation of a synthetic medium containing penicillin on the growth of *Escherichia coli* A.T.C.C. 6522 subsequently inoculated into it. (See text for further explanation.)

of the second set were inoculated and growth in those tubes was followed with the Lumetron. Figure 2 shows that the same general pattern was found in both sets of tubes but that the absolute values seen in tubes inoculated after eight hours of incubation (solid circles) were higher than those at corresponding times in tubes inoculated at the start of the experiment (open circles), as would be expected in cultures containing less penicillin. The curves for the control cultures were identical whether the solutions were inoculated before (open circles) or after (solid circles) preliminary incubation.

[1] Concentrations of penicillin are expressed in terms of molarity to facilitate comparison of the effects of glutathione and of the antibiotic. Those who are more accustomed to thinking in terms of units of penicillin per ml. or of micrograms per ml. will find the following equivalents helpful in converting from one system to the other: 1.25×10^{-6} *M* potassium benzyl penicillin = 4.66 micrograms/ml. or 7.77 units/ml.

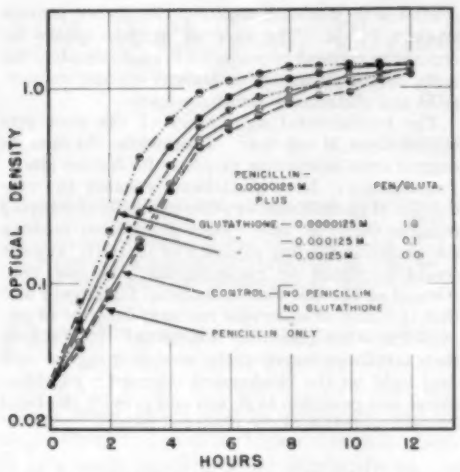
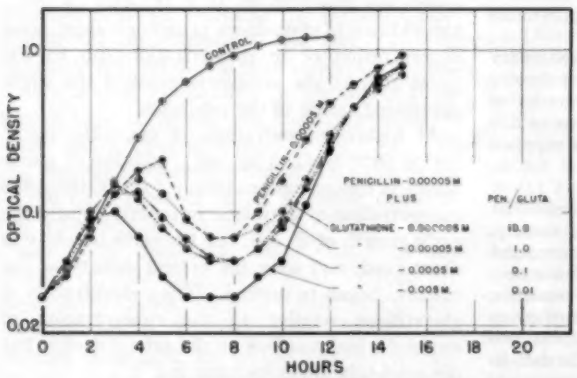


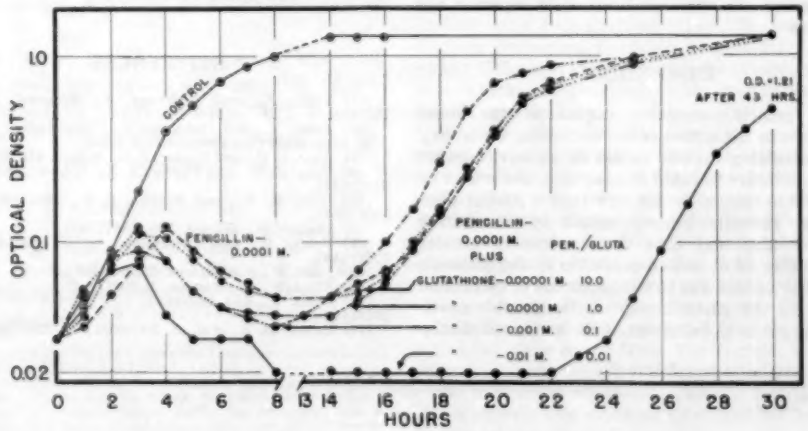
Fig. 3.—Effect of addition of glutathione on the growth of *Escherichia coli* A.T.C.C. 6522 in a chemically defined medium containing potassium benzyl penicillin (1.25×10^{-5} *M* = 7.8 units per ml.).

Periodically during the first eight hours of incubation, samples were removed from one set of each series of tubes containing penicillin, the samples were centrifuged and then filtered through sintered glass, and the penicillin content of each of the resulting sterile filtrates was ascertained by the standard cylinder-plate method. The results of the assays, indicating the per cent of initial potency remaining, are shown near the bottom of Fig. 2. The differences between values for samples from uninoculated and inoculated tubes may be assumed to be a measure of the amount of the antibiotic (*a*) bound by the organisms, and (*b*) inactivated by penicillinase or other metabolic products of the bacteria.

Next, the effect of adding glutathione to cultures containing penicillin was investigated. In any one experiment, a single initial concentration of penicillin was used and the initial molar concentrations of glutathione were varied from 0.1 to 100 times that of penicillin. The range of glutathione concentrations in the different experiments was from 1.0×10^{-5} *M* to 5.0×10^{-3} *M*. Preliminary experiments showed that concentrations from 5.0×10^{-4} *M* down to 2.0×10^{-4} *M* eliminated the brief lag in early growth of *E. coli* in the synthetic medium and increased slightly the rate of logarithmic growth but did not significantly affect the final absorbance that was attained. Lower concentrations and higher concentrations (up to 10^{-3} *M*) of glutathione seemed to exert no effect on growth in the absence of penicillin.

The response of *E. coli* to combined exposure to penicillin and glutathione is shown in Figs. 3–5.





Figs. 4 (left) and 5 (below).—Effect of addition of glutathione on the growth of *Escherichia coli* A.T.C.C. 6522 in chemically defined medium containing potassium benzyl penicillin (5×10^{-5}*M* = 31.3 units per ml.) and (1×10^{-4} *M* = 62.5 units per ml.), respectively.

At a very low concentration of penicillin (Fig. 3), the effect of the antibiotic was completely nullified by glutathione. The stimulating effect of glutathione on early growth seemed to dominate and even the cultures containing penicillin grew more rapidly than the controls which contained no antibiotic and no glutathione. The final absorbance attained, however, was about the same in all cultures.

Evidence of the optimal effect seen with intermediate concentrations of glutathione and referred to above is seen in Fig. 3. Growth was more rapid in media containing 1.25×10^{-4} *M* glutathione than in solutions containing either higher or lower concentrations. It seems as though penicillin had some effect in this connection, because in the absence of penicillin the optimal range of glutathione concentrations was from 2.0×10^{-4} *M* to 5.0×10^{-4} *M* and a concentration of 1.25×10^{-4} *M* glutathione had no measurable effect on growth.

Figures 4 and 5 for penicillin concentrations of 5.0×10^{-6} *M* and 1.0×10^{-4} *M*, respectively, show quite a different effect of simultaneous exposure of organisms to penicillin and glutathione. At both concentrations of antibiotic, glutathione improved slightly the early growth of *E. coli*, partly by minimizing, or even eliminating, the lag period. However, the early benefit was short-lived, and soon the population began to decline. The concentration of glutathione relative to that of penicillin affected the time of onset of decline in absorbance but seemed to have little effect on the rate of decline—the descending portions of the curves in Figs. 4 and 5 are approximately parallel.

For the most part, the duration of the secondary lag periods following the decline in optical density increased as the concentration of glutathione relative to penicillin increased. The only exception to this general phenomenon was seen in cultures supplied with an initial penicillin concentration of 1.0×10^{-4} *M* and a glutathione concentration of 1.0×10^{-3} *M* (Fig 5). It may be merely coincidence that at the same penicillin/glutathione ratio, namely, 1:10, maximal stimulation by glutathione occurred when organisms were exposed to a much lower concentration of penicillin (Fig. 3). This phenomenon was not observed at any of the other concentrations studied, but was observed repeatedly at the two concentrations shown in Figs. 3 and 5. The data in those figures are averages from eight separate experiments.

DISCUSSION

Since penicillin-sensitive organisms are most susceptible to the action of the antibiotic when they are metabolizing rapidly or are in an environment that is conducive to rapid metabolism, one might be prompted to reason by analogy that a similar phenomenon prevails among relatively insensitive organisms also and that the apparently greater susceptibility of *E. coli* to penicillin in the presence of glutathione was due to enhancement of metabolic activity by the glutathione. Although this possibility has not been ruled out, data from preliminary studies with Warburg respirometers do not provide support for it. The rate of oxygen uptake by organisms exposed to penicillin is approximately the same as the uptake by organisms exposed to penicillin and glutathione simultaneously.

The fundamental significance of the data presented above is not clear. Meanwhile, the data do suggest some interesting problems for further study. For example: Does glutathione enhance the production of penicillinase or other penicillin-destroying metabolites by *E. coli*? Does glutathione increase the penicillin-binding capacity of *E. coli*? Does it retard synthesis or reconstitution of some constituent of the cell that is essential for growth and that is bound or otherwise rendered inactive or unavailable when penicillin is present? It is hoped that metabolic experiments now in progress will shed light on the biochemical interaction of glutathione and penicillin in *E. coli* and provide the basis for interpretation of the observed results.

SUMMARY

As part of an investigation of the comparative effects of penicillin on sensitive and on relatively insensitive organisms, the response of *Escherichia coli* A.T.C.C. 6522 to simultaneous exposure to penicillin and glutathione in a chemically defined medium was studied. When the penicillin concentration was 1.25×10^{-6} *M* (4.66 μg./ml.), the addition of glutathione in an equivalent molar concentration or in concentrations up to 100 times that of the antibiotic nullified the slight depressing action of the penicillin.

At higher concentrations of penicillin, up to 1.0×10^{-4} *M* (37.3 μg./ml.), addition of glutathione in concentrations from 0.1 to 100 times the concentration of antibiotic improved slightly the early growth of *E. coli*, but the early benefit was short-lived, and soon the optical density of the cultures began to decline. The concentration of glutathione relative to the concentration of antibiotic had no effect on the rate of decline but did markedly affect its duration.

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A Preliminary Phytochemical Investigation of *Desmodium gangeticum* DC, I*

By B. K. AVASTHI and J. D. TEWARI

Several phytochemical components have been isolated from *Desmodium gangeticum*. The isolation of these principles has been described and a preliminary study of their physical and chemical properties is reported.

Desmodium gangeticum DC (*Leguminosae*, Sub-order *Papilionaceae*), popularly known as Shāparṇi in Hindi, is a well known Indian medicinal plant and is used by Ayurvedic and by Unani physicians as a febrifuge and anticatarrhal. It is a small shrub growing commonly on the lower Himalayas and in the plains of India. The most useful part of the plant is root though other parts such as seeds and leaves are also used medicinally.

As regards the chemical constituents of the plant, Dymock, *et al.* (1), made a preliminary study and have reported the presence of a resin, alkaloid, and oil in the roots. Since then, no reference has been found in the literature concerning the constituents of this drug. It was, therefore, deemed desirable to undertake a complete chemical examination of Shālparṇi roots with a view to isolate and study the different active principles present therein.

A preliminary examination of the roots by us revealed the presence of the following chemical constituents: (1) Lactone, m. p. 82–83°, yield: 0.03%; (2) Alkaloids, liquid bases, yield: 0.05%; (3) Wax, m. p. 58.4°, yield: 0.7%; (4) Resin (ether soluble), m. p. 72°, yield: 2.1%; (5) Phytosterolin, m. p. 278°, yield: 0.02%; (6) Another compound, m. p. 150°, yield: 0.05%; (7) Colouring matter, m. p. above 300°, yield: 0.1%.

Besides these, the presence of an organic acid, saponin, reducing sugars, and essential oil was also detected by qualitative analysis: no pure compound could, however, be isolated for the pursuance of further investigation.

EXPERIMENTAL

Fresh drug (roots) was supplied by M/s. S. R. Gupta & Sons, Saharanpur (India). It was dried and powdered in the laboratory. About 10 Kg. of the crushed material was completely extracted with hot rectified spirit under reflux in lots of 2 Kg. each in a large round-bottomed flask and the extracts were filtered hot. The hot combined filtrate was allowed to stand for five days after which a grayish white substance precipitated. This was filtered, washed several times with cold alcohol and dried in a vacuum desiccator. Yield: 0.1% of dried roots. It was then refluxed with petroleum ether (b. p. 40–60°) over a water bath. The extract on evaporation to dryness yielded a light yellow substance from which on crystallization from absolute alcohol and animal charcoal, white flakes, m. p. 82–83° in 0.03% yield were obtained. The residue which was insoluble in petroleum ether on being refluxed with absolute alcohol produced more of the same compound. The compound was insoluble in water, ether, and methyl alcohol but soluble in petroleum ether (hot), chloroform, benzene, and acetone. It was insoluble in acids and alkalis but decomposed on heating with caustic alkali. It gave no color tests for sterols with usual reagents. It contained only carbon, hydrogen, and oxygen.

Anal.—Calcd. for $C_{16}H_{30}O_3$: C, 75.61; H, 11.81; mol. wt., 254. Found: C, 75.58; H, 11.67; mol. wt. (Rast), 266.

Further work regarding the chemical structure of this compound will form the subject matter of the next communication.

The deep red alcoholic extract, after the removal of the above compound, was concentrated under reduced pressure to yield more of the same compound. Finally the extract was concentrated by distillation to the consistency of a dark brown syrup. On drying it completely in a vacuum desiccator, the total extract left was 9% of the raw material (dried roots). This was lixiviated with acidulated water (1% hydrochloric acid) over a water bath for about six hours, shaking occasionally. The solution was filtered after cooling and the insoluble residue washed two or three times with hot water. Thus the total alcoholic extract was divided into two portions: (*a*) The crude resin fraction remaining insoluble in acidulated water, yield: 7%; and (*b*) The dark colored aqueous solution which contained alkaloids and other water soluble constituents.

Solution (B).—The solution (B) as obtained above was concentrated to half its volume, cooled and filtered. The clear red filtrate was shaken successively with ether and chloroform which removed colored impurities. The remaining liquid was neutralized with dilute ammonia and shaken repeatedly with chloroform until the alkaloids were completely extracted. The combined chloroform extract was washed twice with water, dried over anhydrous calcium chloride, and filtered. On distilling off chloroform from the filtrate, a brown gummy residue was left which consisted mostly of a crude mixture of alkaloids. Yield: 0.05%. The above process was repeated to ensure further purification of the alkaloid.

* Received April 18, 1955, from the Chemical Laboratories, Allahabad University, Allahabad (India).

One of the authors (B. K. A.) wishes to express his indebtedness to the "Kanta Prasad Research Scholarships Endowment Trust" of Uttar Pradesh, India, for providing him a scholarship which has enabled him to take part in this investigation.

The alkaloid gave precipitates with all the usual alkaloid reagents excepting Mayer's reagent. It has a bitter taste and sharp odor, and contains nitrogen as tested by fusion with sodium and addition of ferrous sulfate and ferric chloride followed by dilute hydrochloric acid which produced green coloration. On trying to separate individual alkaloids by means of different solvents, no solid fraction could be isolated from it. Hence it seemed to be a mixture of liquid bases and it was deemed desirable to submit it to fractional distillation under reduced pressure.

Fractional Distillation of Alkaloid Mixture Under Reduced Pressure.—Five grams of the crude alkaloidal mixture was distilled at 0.2 mm. pressure. The fraction boiling from 175–200° was 3 Gms. It was a viscous brown oil mixed with some crystalline material. This was taken up in chloroform and the chloroform solution was concentrated and finally dried in a vacuum desiccator. The residue was treated with dilute ethyl alcohol when a cream colored substance remained undissolved in the solution. After filtering and drying the substance, its melting point was 134°. The alcohol soluble fraction did not yield any solid substance. It was dissolved in a minimum quantity of methyl alcohol and treated with a methyl alcoholic solution of picric acid, when a yellow picrate, m. p. 248°, was obtained which was recrystallized from methyl alcohol.

The remaining alkaline liquid from which chloroform extracted out the alkaloids was concentrated and dilute hydrochloric acid was added when a brown precipitate was obtained. It was purified by dissolving in ammonia and adding dilute hydrochloric acid to the solution thus obtained when a coloring matter (pigment) precipitated, which was washed and finally dried at 100° in an air oven. Yield: 0.1%. It did not melt up to 300°. It is easily soluble in absolute alcohol and fairly so in hot glacial acetic acid. It dissolves in dilute alkalis and is reprecipitated by acids.

The liquid from which most of the coloring matter was thus removed was made neutral with alkali, warmed and treated with an excess of warm aqueous solution of neutral lead acetate. A light yellow precipitate was thrown down. This was filtered, washed with cold water and dried. By working up this precipitate in the usual way, the presence of a phenolic unsaturated acid could be detected by qualitative methods. No pure compound could, however, be obtained from this fraction.

The filtrate left after separating the lead lake was similarly treated with basic lead acetate and the white precipitate thus obtained was collected. It was decomposed by means of hydrogen sulfide and filtered. The filtrate as such did not reduce Fehling's solution but did so after hydrolysis with mineral acids. On shaking with a dilute solution of sodium carbonate, it gave froth characteristic of saponins. Attempts to obtain the saponin or the sapogenin by the hydrolysis of the saponin failed to give any pure compound, though the presence of reducing sugar in the products of hydrolysis was detected.

The mother liquor, after removing the precipitate of basic lead acetate, was freed of lead by hydrogen sulfide and the solution concentrated under reduced pressure when a syrupy mass consisting of reducing sugars was obtained. An osazone was prepared by treating the solution with phenylhydrazine and glacial acetic acid, which after recrystallization from 70% alcohol was found to melt at 204°, unchanged when mixed with a pure sample of glucosazone.

Crude Resin (A).—The water insoluble resin was fractionated with different organic solvents in the following way. It was successively extracted with: (*a*) petroleum ether (40–60°), (*b*) ether, (*c*) chloroform, (*d*) ethyl acetate, and (*e*) amyl alcohol, in the order given, and the extracts were examined separately. Only the petroleum ether and ether extracts have been examined in detail because the quantity of extracts from the other solvents was too small.

(*a*) *Petroleum Ether Extract.*—The crude resin (A) was refluxed with petroleum ether (40–60°) several times and the extracts were filtered hot. The clear filtrate was allowed to stand for four to five days when a brownish substance (i) settled down. It was filtered, washed with the same solvent and dried.

Petroleum ether extract
(cooled and then filtered)

precipitate (i) — mother liquor (ii)

The precipitate (i) was then washed with cold very dilute alcohol to remove associated resin and oily matter. It was then crystallized from 95% alcohol and a cream-colored compound was isolated. After recrystallization from absolute alcohol and a little animal charcoal, the compound melted at 150°. Yield: 0.05%.

Anal.—Calcd. for $C_{18}H_{30}O_3$: C, 71.11; H, 11.11; mol. wt., 270. Found: C, 71.2; H, 10.9; mol. wt. (Rast), 265.7

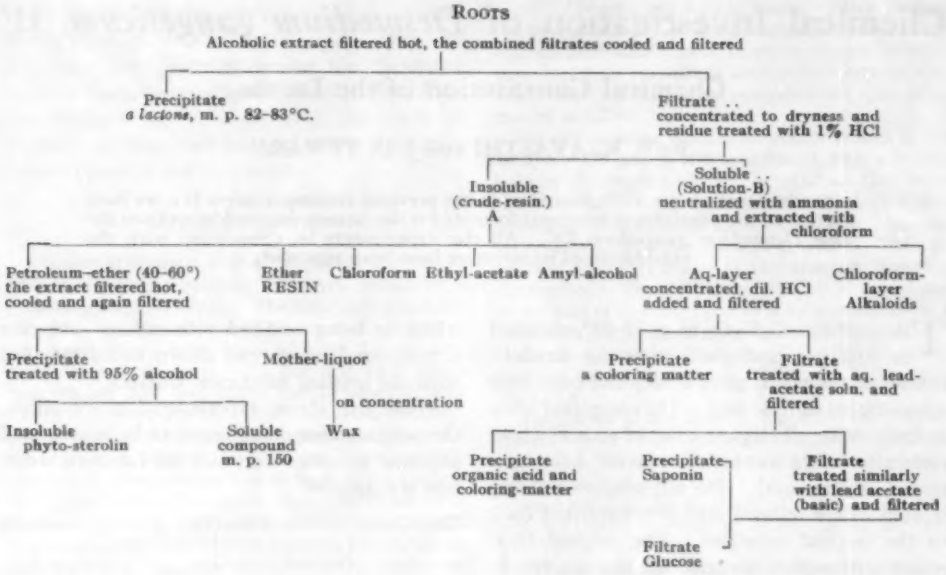
It is also a saturated aliphatic compound and gives intense red coloration with concentrated sulfuric acid. It is insoluble in caustic soda and gives no effervescence with a dilute solution of sodium bicarbonate and with ferric chloride. This could not be further investigated on account of the paucity of the material.

That part of the compound (i) which remained undissolved in alcohol was refluxed with petroleum ether (40–60°). This solution on cooling yielded a colorless compound which after repeated recrystallizations from hot absolute alcohol melted at 278° (with decompn.). On cooling the mother liquor over ice, a further crop of the same compound was recovered. Total yield: 0.02% (on the weight of roots). It gave Liebermann-Burchard color reaction for sterols. It was insoluble in water and sparingly soluble in hot alcohol, chloroform, and benzene.

Anal.—Calcd. for $C_{36}H_{58}O_6$: C, 73.04; H, 10.26; mol. wt., 575. Found: C, 72.78; H, 10.46; mol. wt. (Rast), 558.90; 562.40.

Acetate: m. p. 147°; Digitonide: m. p. 294°.

It is very difficult to be sure of the exact number of hydrogen atoms in a molecule of high molecular weight, and combustion data alone cannot suggest a correct molecular formula for the compound. The molecular formula of this compound can also be written as $C_{36}H_{61}O_6$ without causing any appreciable difference in the percentage of different ele-



ments in the compound. So both the molecular formulas given above are possible for the compound.

This compound has all the characteristics of a phytosterolin; for example—high melting point, low percentage of carbon in its molecule, and the fact that glucose is obtained in the products of hydrolysis by sulfuric acid. But its aglucone could not be isolated in spite of repeated efforts so that finally it cannot be definitely said which sterol is combined with glucose to form the phytosterolin under examination. The indicated molecular formula of the phytosterolin agrees with that of either a stigmasterol glucoside or sitosterol glucoside.

From the mother liquor (ii), petroleum ether was completely distilled off and a greenish wax was left behind in 0.7% yield. On heating it with benzene and animal charcoal, and distilling off the solvent from the filtrate, a brown wax was obtained. This was dried first in a vacuum desiccator and then in an air oven under reduced pressure. It is soluble in ether, benzene, and alcohol. It has been worked up for its chemical characteristics and for its acid and alcohol components. Further work on the wax will form the subject matter of a separate communication.

(*b*) *Ether Extract.*—The crude resin left undissolved in petroleum ether (40–60°) was repeatedly shaken with ether which dissolved out 30% of the total crude resin. The extract was freed completely of ether when a brown bitter solid was obtained. It was taken up in ether and reprecipitated with petroleum ether. It was also soluble in dilute alkalis from which it could be precipitated unchanged by dilute acids. It was again refluxed with 95% alcohol and activated charcoal to ensure further purification. The final product so obtained after drying was a red brittle solid, m. p. 72°. Further work to elucidate its structure is in progress.

By steam distillation of fresh powdered roots, a light yellow volatile oil was obtained which had a pleasant odor. It was isolated by taking up in ether. As the quantity of the essential oil was very small, no further work could be done.

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Chemical Investigation of *Desmodium gangeticum*, II*

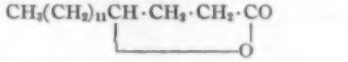
Chemical Constitution of the Lactone

By B. K. AVASTHI and J. D. TEWARI

In continuation of our work described in the previous communication (1), we have discovered the provisional structural formula for the lactone isolated by us from the plant *Desmodium gangeticum* DC. All the experiments in connection with the elucidation of its structure have been reported.

THE LACTONE, $C_{16}H_{30}O_2$, m. p. 82–83°, obtained in 0.03 per cent yield from the alcoholic extract of the plant, gives a deep red color with concentrated sulfuric acid. The compound when hydrolyzed by alkali gave a salt of an acid whose molecular weight was found to be 267.4 (by ignition of its silver salt). The salt solution on being treated with a mineral acid gets converted back to the original substance. The original compound neither gives effervescence nor dissolves in sodium carbonate nor caustic soda solution. It gives no color with ferric chloride solution, neither acts on bromine water nor shows any reaction with cold potassium permanganate solution. It burns with a nonsmoky flame. The substance on being heated with absolute alcohol and concentrated sulfuric acid gives a neutral solid substance, apparently an ester, m. p. 56°. Ultimate analysis and molecular weight determination of the product gives the formula of the substance to be $C_{18}H_{36}O_3$. The original compound on being acetylated as given in the experimental section gives an acetylated product which melts at 130–131°. Elementary analysis and molecular weight determination of the product gives the formula $C_{18}H_{34}O_4$. From it the determination of the hydroxyl group in the original compound gives the value one. This fact together with the acetylation result of the ester obtained from the original compound points out clearly that the compound does not contain any free hydroxyl group in the molecule and the acetylated product is obtained by the fission of the original molecule during the reaction. The reversibility of the original compound and its ethyl ester is proved by converting the original compound into its ethyl ester as stated above. The ethyl ester thus obtained on being heated under reflux with caustic potash in absolute alcohol for six hours, yielded the sodium salt which on being acidified with sulfuric acid gave a compound which was shown to be identical with the original substance, $C_{16}H_{30}O_2$.

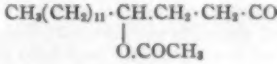
From the above experimental observations, the original compound seems to be a saturated aliphatic γ-lactone for which the following structure is suggested:



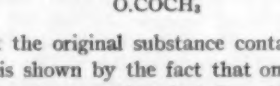
$C_{16}H_{30}O_2$

which is apparently a lactone of the γ-hydroxy palmitic acid. Considering this structure for the original lactone, all the reactions mentioned above can be satisfactorily explained in the following way.

Esterification of the lactone gives the compound $C_{18}H_{36}O_3$, $CH_3(CH_2)_{11}\cdot CHOH\cdot CH_2\cdot CH_2\cdot COOC_2H_5$. The compound obtained on acetylating the original lactone has the structure:



whereas the compound obtained on acetylating the ester is assigned the structure:



That the original substance contains a chain of C_{16} is shown by the fact that on reducing it with phosphorus and hydroiodic acid in a sealed tube at about 120–130°, a syrupy brownish liquid containing iodine was obtained. The reduction of this iodo compound with amalgamated zinc in acid medium gave palmitic acid.

The oxidation of the compound has not yet been done; the oxidation results together with the synthesis of γ-hydroxy palmitic acid will prove the constitution of the lactone obtained from the roots of *Desmodium gangeticum* conclusively.

In two other plants belonging to the *N. O. Leguminosae*, the presence of lactones has been reported by other investigators but they have not been worked out for their structures in detail.

* Received April 18, 1955, from the Chemical Laboratories, Allahabad University, Allahabad, India.

One of the authors (B. K. A.) wishes to express his indebtedness to the "Kanta Prasad Research Scholarships Endowment Trust" of Uttar Pradesh, India, for providing a scholarship which has enabled him to take part in this investigation.

Dutt and Gupta (2) have isolated a lactone, linifoline, m. p. 96°, from the plant *Indigofera linifolia*. They have suggested the molecular formula $C_{28}H_{60}O_2$ for the compound. Rao, Rao, and Seshadri (3) obtained from the seeds of *Psoralia cordifolia* two lactones which they have named Psoralen and Isopsoralen.

EXPERIMENTAL

Alkaline Hydrolysis.—The compound was heated with 2 *N* aqueous solution of caustic potash on a sand bath for three hours. The substance gradually dissolved and the solution so obtained at the end of the experiment, was filtered. The filtrate was divided into two parts. To the first part dilute hydrochloric acid was added and a brownish substance was precipitated. After filtration and drying, it was found to melt at 82°, indicating that it is the starting substance. To the other part, a clear solution of silver nitrate was added and the precipitate (which gradually turned brown on exposure to light) was filtered, washed and dried. On igniting a known quantity of this silver salt in a silica crucible and weighing the residual silver, the molecular weight of the compound was found to be 267.4 (calcd. for $C_{16}H_{32}O_3$: 272).

Preparation of Ethyl Ester.—The original lactone was refluxed with four to five times the amount of absolute alcohol saturated with sulfuric acid on a water bath for twelve hours in order to ensure complete esterification. From the solution, alcohol was distilled off and the residue was shaken with ether. From the ethereal solution the ethyl ester was isolated in light brown flakes, m. p. 56°. It was crystallized from 95% alcohol.

Semimicro combustion analysis gave the following data: Found: C, 72.3; H, 11.8; mol. wt., 296; Calcd. for $C_{18}H_{36}O_3$: C, 72.0; H, 12.0; mol. wt., 300.

The ester as obtained above was dissolved in absolute alcohol and treated with an alcoholic solution of caustic potash. It was then refluxed for six hours on a water bath. From the resulting solution alcohol was distilled off and the residue taken up in distilled water. To this solution dilute sulfuric acid was added and the precipitated substance was extracted with ether. The ethereal solution yielded a compound, m. p. 82°, identical in every respect to the original compound.

Preparation of Acetyl Derivative of the Original Compound.—Five tenths gram of the compound was acetylated with 10 cc. of acetic anhydride using pyridine (0.5 cc.) as the condensing agent. The reaction mixture was refluxed for twelve hours on a sand bath using an air condenser. After cooling, the precipitate was filtered, washed first with dilute hydrochloric acid several times to remove pyridine and then with very dilute sodium bicarbonate solution. The product when recrystallized with ether melted at 130–31°.

To estimate the number of acetyl groups in the acetylated product, it was refluxed with a known volume of alcoholic alkali. After about four hours the excess alkali was titrated with standard hydrochloric acid. A blank experiment was also performed with the same amount of alkali. The percentage of acetyl group in the molecule was thus determined. From this, the number of acetyl and hence that of hydroxyl groups is determined to be 1.

Elementary analysis gave the following results:

Anal.—Calcd. for $C_{18}H_{34}O_4$: C, 68.79; H, 10.82; mol. wt. 314: Found: C, 68.94; H, 11.14; mol. wt. 319.6.

Acetylation of the Ester.—The ethyl ester as obtained above was similarly acetylated and the acetylated product as isolated from the reaction mixture melted at 62°.

Anal.—Calcd. for $C_{20}H_{36}O_4$: C, 70.17; H, 11.11; mol. wt., 342: Found: C, 70.04; H, 11.18; mol. wt., 337.

Reduction of the Original Compound.—The reduction was carried out in a Carius tube with hydroiodic acid and red phosphorus. Five tenths gram of the compound, 3 Gm. of hydroiodic acid (57%) and about 1 Gm. of red phosphorus were introduced into a Carius tube and the farther end sealed properly. It was then placed in the furnace and the temperature was kept constant at about 120–130°. The heating was continued for five to six hours. The tube was allowed to cool and opened carefully. The contents of the tube were extracted with ether and the ethereal solution filtered. The clear ethereal liquid was washed with distilled water till the washings did not give any precipitate with silver nitrate. The ethereal solution on being dried over anhydrous sodium sulfate and evaporation of the solvent yielded a syrupy brownish liquid containing iodine.

The iodo compound on being reduced with granulated zinc by the usual method gave an acid which after crystallization from acetone melted at 61–62° and was identified as palmitic acid, confirmed by mixed melting point with a genuine sample of palmitic acid.

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A Method for Removal of Blood Group A Substance from Pepsin*

By R. Y. GOTTSHALL, ERMA HILL, and ZELMA OZOLINS

A method is described to remove blood group A substance from pepsins used for the removal of nonantitoxic protein from antitoxins. The method was effective in all six manufacturers' preparations tested.

THE METHODS USED at the present time for purification of antitoxins make use of enzymes for the removal of much of the nonantitoxic protein.

The procedures generally employed are those described by Parfentjev (1) and Pope (2), which employ the enzyme, pepsin. This enzyme, in the grade usually used, however, contains as an impurity, human blood group A substance. None of the preparations tested were found to contain blood group B substance. The blood group A substance when added to the antitoxic plasma with the pepsin is not removed during subsequent purification and concentration of the antitoxin.

All of the commercial pepsins we have tested thus far, except one of the two preparations of twice crystallized pepsin, were found to contain more than 0.002 mg. of blood group A substance per mg. of pepsin. Because under certain conditions injection of these substances could be followed by a rise in titre of isohemoagglutinins, administration of antitoxins and other biologics free from them would be highly desirable.

Fenger and Andrew (3) made a preliminary purification of gastric mucosa by precipitating the enzyme between acetone concentrations of 50 and 75 per cent previous to isoelectric purification. Morgan and King (4) found that treatment of a purified blood group A preparation with acetone in a concentration of between 39 and 50 per cent gave rise to two fractions.

When 1:10,000 pepsin was treated with 50 per cent acetone, the precipitate discarded, and acetone added to the filtrate to a concentration of 75 per cent, we have found that with most preparations the blood group A substance was removed with only a moderate loss of enzyme activity.

METHOD

The method is as follows: A 10 or 15% aqueous solution of pepsin is adjusted to pH 3.0 with 0.2 *M* sulphuric acid. After cooling to 5° 1% filter cell[1] is added and the suspension is filtered through paper. An equal volume of cold acetone is added to the filtrate with constant stirring. The mixture is left at 5° for fifteen to eighteen hours when much of the precipitate will have settled. The supernatant is decanted, 1% filter cell added, and filtered through paper under positive pressure. To the clear filtrate is added, with stirring, an equal volume of cold acetone. The precipitate is allowed to settle and the clear supernatant drawn off and discarded. The precipitate is centrifuged at 5° and dissolved in a small quantity of distilled water. One gram of filter cell is added per 100 ml. of liquid prior to filtration through paper. The clear filtrate is dried from the frozen state and stored as the dry powder.

Six preparations made by three different manufacturers have been processed. The results are given in Table I. Only the blood group A substance from pepsin No. 6 could not be removed with 50% acetone. Fifty-five per cent, however, was effective in eliminating this substance. No evidence of blood group B substance could be found in any of the purified preparations.

Pepsin was assayed according to the N. F. test (5). Tests for blood group A and B substances

TABLE I.—ASSAY AND AMOUNT OF BLOOD GROUP A SUBSTANCE OF LOTS OF PEPSIN BEFORE AND AFTER FRACTIONATION WITH ACETONE

Pepsin No.	Fraction, %	Assay		Recovery, %	Amount of Group A Substance, mg./mg. of Pepsin	
		Before Purification	After Purification		Before Purification	After Purification
1	50–75	1:10,000	1:17,500	80	0.03	0.0[a]
2	50–75	1:8,000	1:15,000	85	0.03	0.0
3	50–75	1:10,000	1:20,000	100	0.003	0.0
4	50–75	1:10,000	1:15,000	74	0.03	0.0
5	50–75	1:10,000	1:20,000	90	0.03	0.0
6	50–75	1:10,000	.....	..	0.08	0.0001
	55–75	1:10,000	1:20,000	62	0.08	0.0

a Less than 0.00001 mg. per mg. of pepsin.

* Received August 19, 1955, from the division of Laboratories, Michigan Department of Health, Lansing.

[1] Celite, Supercell, Standard. Johns-Manville Corporation, New York, N. Y.

were made by a technique based on inhibition of hemagglutination.

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The Assay of Sodium Radio-iodide (I^{131}) Used for Medicinal Purposes*

By JOHN J. PINAJIAN† and JOHN E. CHRISTIAN

The calibration of I^{131} using the electroscope, the method of absolute beta counting, and the method of the simulated I^{131} reference source is reported. The characterization of the beta radiation of I^{131} through the determination of the mass absorption coefficient in aluminum is described. A test for the radiochemical purity is given.

IODINE-131 has increased in medicinal importance since the last revision of the U. S. P. (1) as substantiated by a number of literature references (2–8). Our object has been to evaluate methods of assay of I^{131} for the establishment of standard techniques and to promote the development of a standard medicinal radio-iodide (I^{131}) to be within the scope of the U. S. P. Unfortunate as it may be, the more refined techniques (e. g., 4 π counting, coincidence counting, scintillation counters, tetramethyl lead counters, and ionization chamber measurements) are either not sufficiently developed commercially or the design of the apparatus has not been standardized. The general principles of assay and the fundamentals of the physical problems of the medicinal applications of artificially radioactive substances have been discussed by Mayneord (8), Gray (9), Seliger and Schwebel (10), Manov (11), Putman (12), Perry (13), Evans (14), and Marinelli, Quimby, and Hine (15). Assay methods similar to those adopted for U. S. P. XV had been developed by G. I. Gleason and D. L. Tabern at Abbott Laboratories prior to the initiation of this work and the authors have had their full cooperation at all times.

* Received February 11, 1955, from the Bio-Nucleonics Laboratory, Purdue University, School of Pharmacy, Lafayette, Ind.
Presented before the Scientific Section, A. PH. A., Boston meeting, August, 1954.
This work resulted in the major part of the monograph for Sodium Radio-iodide (I^{131}), U. S. P. XV.
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CALIBRATION USING THE ELECTROSCOPE

The principle of operation of the electroscope is given by Lauritsen and Lauritsen (16) and the principles of its use in calibration has been discussed elsewhere (3, 7, 15).

A Lauritsen quartz-fiber electroscope[1] was mounted on a plywood board so that the axis of the aluminum can was perpendicular to the length of the board. The board itself was mounted a meter from the concrete flooring. A vacuum tube rectifier was used as the source of charge. The light source was at a distance of 10 meters and was turned on only when readings were taken. The area was cleared of all objects which would contribute to scattering. Prior to the initial measurement of the day, the electroscope was first charged and discharged to minimize the soaking effect of amber. All measurements were made from center-of-sample to center-of-electroscope.

In a number of survey meter type of electroscopes available for such measurements, the physical center of the can is difficult to determine. The concept of the electrical center of the instrument is useful for this reason. The drift of the quartz fiber of an electroscope is not linear over its entire range. Unless drift time measurements are always taken between the same two points on the scale, that portion of the scale which is linear must be experimentally determined.

Measurement of the Linearity of the Scale of the Electroscope.—A radium needle was mounted at 50 cm. and the electroscope charged to ~10 units below the zero mark. The time was noted as the fiber passed each fifth division mark from 0 to 100. This was repeated at 75 cm. A plot of the rate of drift as a function of distance showed, in each case, linearity of the scale of the electroscope from 0 to 60 units.

[1] Manufactured by Fred C. Hensen Co., Pasadena, Calif.

Measurement of the Electrical Center of the Electroscope.—A radium needle was moved successive distances, d, from the outside of the can and drift rates, R, were determined between 40 and 60 on the scale. Corrections for background were made. The results were plotted as $1/\sqrt{R}$ as a function of d. Extrapolation of the curve placed the electrical center ~3.0 cm. from the outer edge of the can. This compared favorably with the 2.9 cm. distance to the physical center.

Standards.—A radium needle with 0.5 mm. Pt shield was calibrated against a particular sample of I^{131} from Oak Ridge. Co^{60} activity in 2 ml. of 0.0015 *M* $CoCl_2$ solution, contained in a 10 ml. multiple dose vial capped with a rubber sleeve was calibrated against the radium.

Preparation of Samples.—All glassware used was previously treated with a diluting solution containing 0.02 *M* NaOH, 0.005 *M* $NaHSO_3$, and 0.0015 *M* KI. A quantity of I^{131} activity was used which would give approximately the same rate of drift as the standard in each case. The I^{131} activity was transferred with a micropipet to a 10-ml. multiple-dose vial, the two rinses of the pipet added, and the solution brought to approximately 2 ml. The vial was capped with a rubber sleeve. When necessary, appropriate dilutions were made with the diluting solution.

Calibration of the Samples.—Each sample was placed 100 cm. from the center of the electroscope and the time of drift of the fiber determined from the 20 to the 60 position of the scale. The rate of drift was calculated and the background rate of drift subtracted. The relationship of the unknown, *U*, to the standard, *S*, may be expressed

$$U = \frac{R_u}{R_s} \cdot A_s \cdot \frac{I_{\gamma s}}{I_{\gamma u}} \text{ mc.}$$

where R_s and R_u are the rates of drift of the standard and unknown respectively, $I_{\gamma s}$ and $I_{\gamma u}$ are constants for the standard and unknown respectively, and A_s, the millicurie strength of the standard. I_γ expresses the exposure in roentgens per mc./hr. at 1 cm. in standard air. I_γ was calculated for gamma ray energies from 0.01 to 4.00 Mev. to 5 places and plotted on a sheet of millimeter graph paper 3 x 5 feet. Calculations were made using 4 places and I_γ was evaluated to 2 places. [It should be noted that I_γ does not include the contribution due to X-rays arising from internal conversion. This correction has been determined experimentally by analysis of an absorption curve of the total radiation in copper and amounts to about 1.5% for I^{131} (17). The contribution due to bremsstrahlung is in part included in this correction.]

The evaluation of I_γ permits rapid determination of the millicurie strength of gamma-emitting radioisotopes of known disintegration schemes by simply measuring the relative ionization of a sample with that of a standard. The calculated values of I_γ for I^{131} (based on the decay scheme of Bell and Graham (18)) and for Co^{60} [decay scheme, (19–22)] were estimated to be 2.36 and 13.52 respectively. The mean of the corrected results of gamma-ray output through 0.5 mm. Pt reported by Ghosh, Kastner, and Whyte (23) as 8.41 $rmc^{-1}hr.^{-1}$ at 1 cm. was used. Necessary corrections for decay were made using a detailed I^{131} decay correction table (24). The evaluation of this procedure is given in Table I.

CALIBRATION BY ABSOLUTE BETA COUNTING

A 2.70 mg./cm.[2] mica end-window Geiger-Müller tube[2] was mounted on a Technical Associates TM-7 tube mount and sample tray holder[3] and set in a 1³/₄-inch lead shield.[3] The inner walls and doors were lined with brass and aluminum. The tube mount and sample trays were made of Lucite. A hole with a ridge was machined in the center of the sample tray to accommodate sample holders (aluminum cups, nickel plated steel cups, stainless steel cups, and iron washers). A ¹/₈-inch leaded brass plate with a defining orifice, coaxial with the anode wire and with a 14-degree bevel, was placed on shelf 1.

This technique was first introduced by Elliott, Engelkemeir, and Rubinson (25) and later developed by Gleason, Taylor, and Tabern (26). Putman (12) has modified the arrangement by the use of a brass iris.

An expression for the disintegration rate may be written

$$D = \frac{N}{G \cdot f_b \cdot f_a \cdot f_h \cdot f_s \cdot f_w} \qquad (2)$$

where

- D = absolute number of disintegrations per minute
- N = observed counts per minute $(n) \cdot f_\tau$ minus the background
- G = calculated geometry
- f_τ = factor for the effect of the finite resolving time of the Geiger-Müller tube and circuit
- f_b = factor for the increase in counting rate due to backscattering by the material supporting the source
- f_a = factor for the effect of air in scattering beta particles into the counter
- f_h = factor for the effect of the source support structure and walls of the housing and shield in scattering beta particles into the counter
- f_s = factor for the effect of the mass of the source in causing scattering and absorption of beta particles
- f_w = factor for the effect of the beta particle absorption by the counter window and the air in the space between the source and the tube

A number of workers have discussed one or more of these factors in some detail (25–32). In order to determine the disintegration rate of a beta emitter, each term in Eq. 2 must be evaluated.

G: The geometry of this arrangement was calculated to be equal to 0.0158. Dried sample mounts varied in diameter from ~3 mm. in the case of Ra-DEF to 6–8 mm. in the case of I^{131}. The first three terms of the Blachman equation (27) were used to determine the geometry, i.e.,

$$G = 0.5 \left\{ 1 - \frac{1}{(1+\beta)^{1/2}} - \frac{3}{8} \frac{\beta\gamma}{(1+\beta)^{5/2}} - \ldots \right\} \qquad (3)$$

where

[2] TGC-1 Tracerlab Inc., Boston, Mass.
[3] Technical Associates, Glendale, Calif.

$$\beta = \frac{b^2}{a^2} \text{ and } \gamma = \frac{c^2}{a^2}$$

and a = distance from source to brass collimator, b = radius of the orifice, and c = radius of the source. A plot was made of the geometry as a function of the source radius and the geometry value for a particular source of radius c was taken from the plot. It can be shown, for this arrangement, that for sources of radius ≤ 1.8 mm. no significant error will be introduced by using $G = 0.0158$.

f_r: The true counting rate (N) of a sample may be expressed as

$$N = \frac{n}{1 - n\tau} \tag{4}$$

where n is the observed counting rate and τ is the resolving time of the Geiger-Müller counter in the particular circuit. For convenience, a correction factor, f_r, where

$$f_r = \frac{N}{n} = \frac{1}{1 - n\tau} \tag{5}$$

may be used.

The determination of the resolving time was made with a pair of sources, using the following corrected (33) equation (34)

$$\tau = \tau_1 \left\{1 + \frac{\tau_1}{2}(n_{12} - 3b)\right\} \tag{6}$$

where

$$\tau_1 = \frac{x}{2(n_1 - b)(n_2 - b)}$$

and

$$x = n_1 + n_2 - n_{12} - b$$

The subscripts refer to the individual sources counted individually (n_1, n_2) and collectively (n_{12}) and the background is denoted by b. If source n_1 is approximately equal to source n_2, the best distribution of the counting time, t, can be shown to be

$$t_{12} = \sqrt{2}t_1 \tag{7}$$

where, again, the subscripts refer to the total time the sources are counted individually (t_1) and collectively (t_{12}). Furthermore, it can be shown that for a tube having a resolving time of the order of 200 μsec. and counting each source for ten minutes, the counting rate per source should be about 14,140 c. p. m.

For a given resolving time, using Eq. 5 a plot was made of f_r as a function of the counting rate. Since, whenever convenient, counts were made so that the total count was at least 10,000 (i.e., $\geq 160 \times 64$), only the register readings were taken (after depression of the interpolation switch); the counting rate in the plot was scaled to 64.

The mechanical register of the scaling circuit was checked for losses due to the resolving time of the register. These losses were found to be incurred when it reached ~700 register counts per minute (i.e., ~45,000 c. p. m.).

f_b: Sources covering an area ≤ 0.5 cm.^{2} were prepared by depositing identical aliquots on thin Zapon films, aluminum cups, stainless steel cups, and nickel-plated steel cups.

The thin Zapon films were prepared by placing ten drops of Zapon Solution[4] on the surface of clean water in a large dish. The solution was allowed to spread over the surface and the solvent was allowed to evaporate. A semicircular loop was dipped under the surface and moved to a position beneath the film. The leading edge of the loop was raised at the center of the film so that a double layer of film was draped over the loop. The film was brought down over an iron washer such that the washer had a double layer of film adhering to the surface. The water was evaporated from the film by placing it under a heating lamp for ten minutes at 12 inches. The density of these films was determined by depositing 10 double layers of film over a tared Lucite loop. The loop was dried under a heating lamp after each deposition and placed in a desiccator for one hour after the last drying process. Excess film was trimmed off. The weighing was performed on a micro balance. The density of a double layer of film was found to be 21 μg./cm.^{2}

Constant geometry was maintained and the counting rates were determined for each of the samples. From these data, after corrections for resolving time and background were made, the backscatter was calculated from

$$f_b = \frac{\text{counting rate with metal backing}}{\text{counting rate with no backing (on film)}} \tag{8}$$

Assuming f_b for film mountings to be 1.00, the following values were found:

Aluminum cups	1.30
Stainless steel cups	1.43
Nickel plated steel cups	1.48

f_a: The factor for the effect of air in scattering beta particles into the counter is only appreciable when dealing with soft beta radiation traversing a large distance. Since the scattered beta particles form such oblique angles that they are not likely to pass through both the orifice and the counter window, assuming f_a to be equal to unity would not introduce an appreciable error.

f_h: The effect of scattering from the source support structure and walls of the housing has been shown to be negligible (27) in a similar type of housing and again, the use of the defining orifice on shelf 1 with the source on shelf 3 tends to reduce this already negligible factor so that an assumption of unity would be valid.

f_s: The factor for the effect of the mass of the source in causing both scattering and absorption of beta particles was assumed to be unity since the sources used were essentially weightless (i.e., <0.1 mg./cm.^{2}).

f_w: The evaluation of the effect of absorption was made by extrapolation of an aluminum absorption curve to zero total absorber. The absorber was placed close to the window (i.e., laid on top of the brass collimator) to minimize scattering into the counter. The magnitude of the error introduced by failure to do this was demonstrated by placing a source (*Ra-DEF*) on shelf 3 and placing an absorber on shelf 1. When another absorber of the same

[4] A satisfactory solution may be had by soaking ~5 Gm. purified pyroxylin in 1 oz. amyl acetate overnight with occasional shaking. Decant solution of dissolved material into a dropper bottle for use.

thickness (6.96 mg./cm.²) was placed directly over the source, an increase of ~9% was noticed in the counting rate.

A series of aluminum absorbers were used in the range of 1.85 to 70.0 mg./cm.². The net beta activities with the various absorbers used were obtained by subtracting the activity found with a 250 mg./cm.² or greater absorber. The net beta activity was plotted on semilog paper as a function of the total absorber thickness. The total absorber thickness is the thickness of the Geiger-Müller window (as stated by the manufacturer) plus the air equivalent thickness, all expressed in mg./cm.². An extrapolation of the absorption curve (which was linear from 10 to 30 mg./cm.²) was used to determine the counting rate at zero absorber. The absorption factor f_w is the ratio of the counting rate under the experimental conditions to that at zero total absorber, i.e.,

$$f_w = \frac{\text{counting rate under the experimental conditions}}{\text{counting rate at zero total absorber}} \quad (9)$$

For a point source, mounted on Zapon film, the value of f_w thus determined was within 0.5% of the calculated value based on the beta spectrum given by Bell and Graham (18) and the expression given by Gleason, Taylor, and Tabern (26) relating the mass absorption coefficient and the maximum beta energy:

$$\mu = 0.017 E_{max.}^{-1.43} \quad (10)$$

and since

$$w = \frac{N_t}{N_0} = \alpha e^{-\mu t} + \beta e^{-\mu t} + \gamma e^{-\mu t} + \delta e^{-\mu t} \quad (11)$$

where N_t = corrected counting rate observed with thickness t between the source and the sensitive volume, N_0 = true beta counting rate at zero thickness, μ = mass absorption coefficient in cm.²/mg., t = total mass per unit area of the distance between the source and the sensitive volume in mg./cm.², and α, β, γ, and δ are the fractional contributions of the various beta energies. The value of f_w used for I^{131} sources of radius $\leq$1.8 mm. was 0.724.

Efficiency of the Counter Using Ra-DEF.—The corrected efficiency of the arrangement described was determined independently by counting calibrated aged *Ra-DEF* sources.

$$Ra\text{-}D \xrightarrow{\beta} Ra\text{-}E \xrightarrow{\beta} Ra\text{-}F \xrightarrow{\alpha} Ra\text{-}G$$

Using subscripts 1, 2, and 3 for *Ra-D*, *Ra-E*, and *Ra-F* respectively, it can be shown that the ratio of the disintegration rate of *Ra-F* (D_3) to the disintegration rate of *Ra-E* (D_2) is

$$\left(\frac{D_3}{D_2}\right)_\infty = 1.017 \quad (12)$$

Ra-DEF samples, mounted on Zapon films and in radioactive equilibrium, were counted in a parallel plate counter. Necessary corrections for resolving time, reflection of alpha particles, and background were made. The alpha disintegration rate (D_3) was determined and from (12) the beta disintegration rate (D_2) was calculated. The factor f_w was experimentally determined by screening out the alpha particles of *Ra-F* and the weak beta particles of *Ra-D* with 5 mg./cm.² of aluminum. Here $f_a = f_b = f_h = f_s = 1.00$. The evaluation of the corrected efficiency of the Geiger-Müller tube with two calibrated *Ra-DEF* sources gave 0.01567 which is in close agreement with the physical measurement of 0.01579 for the geometry.

Vertical and Lateral Displacement of the Source.—It was found that on shelf 3 a point source (I^{131}) could be laterally moved as much as 4 mm. from the center position without decreasing the observed counting rate by more than about 1%. On the other hand, a vertical displacement of 1 mm. caused ~4% change in the counting rate.

Preparation of Samples and Sample Analysis.—Dilutions (using the dilution solution described earlier), if necessary, were made so that the counting rate of the prepared sample was in the order of 2,000 to 5,000 c. p. m. The aliquot (20λ) was transferred by micropipet to a sample mount and 2 rinses of the micropipet were added. One drop of acid silver nitrate solution (1 Gm. $AgNO_3$/liter and 2 drops HNO_3) was added and the material was dried under an infrared lamp at a distance of 12 inches. Samples were analyzed in duplicate. The samples were counted on shelf 3 with the brass collimator on shelf 1.

The observed counting rate was corrected for resolving time losses and the background subtracted. With the evaluation of G, f_w, f_a, f_h, and f_s, Eq. 2 simplifies to

$$D = \frac{N}{0.0158\ 0.724\ 1.00\ 1.00\ 1.00\ f_b} \quad (13)$$

and with appropriate value for f_b, the disintegration rate D was calculated. This was adjusted with a dilution factor to yield disintegrations per milliliter. Necessary corrections for decay were made using a decay table (24). The results are shown in Table I.

CALIBRATION USING THE SIMULATED I^{131} REFERENCE SOURCE

The synthetic standard for I^{131} (i.e., the simulated I^{131} reference source[5]), similar in the beta energy spectrum of I^{131} has been available for several years. Such a standard is designed to meet the need of having a standard such that the detection apparatus will respond in an almost identical manner to the reference standard and the sample. The standard consists of isotopic mixtures of long half-life which, over at least a substantial part of the energy spectrum, has almost identical absorption characteristics to I^{131}.

The addition of a small amount of Co^{60} to Cs^{137} in secular equilibrium with Ba^{137m} results in a mixture which is essentially beta isoenergetic with I^{131} over an absorber range of 10 mg./cm.² to 60 mg./cm.².

The effective beta ray strength equivalent to a given number of millicuries of I^{131} is based upon a calibration against duplicate master standards. These two master standards, in turn, were calibrated by comparing their counting rate with a National Bureau of Standards I^{131}-sample in triplicate. Corrections for decay of the standard are given by the manufacturer for a period of five years.

[5] Tracerlab Inc., Boston, Mass.

By placing an absorber (aluminum) over the sample and taking measurements with a Geiger-Müller counter (the window thickness plus the air equivalent and aluminum absorber thickness should exceed 10 mg./cm.^{2} and be less than 40 mg./cm.^{2}) the ratio of the I^{131} counting rate to that of the simulated reference standard renders an accurate calculation of the strength of the sample.

Preparation of the Samples.—Previous to usage, copper planchets were cleaned by immersion in boiling benzene for two minutes, then soaking in warm benzene for five minutes, drying with gauze, soaking in warm 2 *N* HCl for five minutes, rinsing five minutes, rinsing 5 times with distilled water, and drying with gauze. One drop of 1.0% aqueous solution of Aerosol® AY[6] was spread with the aid of a rubber policeman over the surface of the planchet (leaving a free border of ~1 mm.) and dried. This gave the source a circular area of ~3 cm.^{2}

The sample was diluted with 0.001 *M* KI of *p*H 9. A micropipet was used to transfer the sample (usually 20 λ). Two rinses of the pipet were added to the original deposition. One drop of 0.1% acid silver nitrate solution was added and the sample dried under an infrared lamp at 12 inches. The sample was mounted on a 1-inch aluminum disc sample mount, covered with 1 inch diameter dural foil absorber (6.75 mg./cm.^{2}), and locked into position with the absorber ring.

Analysis of the Sample.—The radioactivity assay was made by placing the simulated I^{131} standard source on shelf 2 and counting with an aluminum absorber of 341 mg./cm.^{2} on shelf 1. The next count was made with the standard absorber (10.4 mg./cm.^{2}) replacing the former absorber. The latter count was made over a sufficient time to yield $\sigma \leq 1$ per cent. The assay of the sample was made in a similar fashion, using the same shelf, identical absorbers, and with the counting rate determined in the same order. The effective net counting rate of the activity in each case is the gross counting rate (i.e., with the standard absorber) minus the effective background (i.e., with 341 mg./cm.^{2} absorber). The millicurie-strength of the sample is equal to the effective net counting rate of the sample divided by the effective net counting rate of the simulated I^{131} reference source, times the effective beta ray strength of the reference source, times the decay factor for the reference source. The result is then adjusted for dilution. Necessary decay correction factors were obtained from a table (24). The results are shown in Table I.

TABLE I.—RESULTS OF I^{131} CALIBRATION EXPRESSED IN TERMS OF PER CENT OF CALIBRATION REPORTED BY SUPPLYING AGENCY

Sample	Method		
	Electroscope	Absolute Beta Counting	Simulated I^{131} Reference Source
ORNL-1	...	103.0	...
ORNL-2	106.1	107.0	98.2
ORNL-3	105.6	...	92.9
ORNL-4	99.8	104.1	99.4
	100.3	95.1	
		104.8	
		104.6	
NBS		105.2	97.5
		104.2	96.0

MASS ABSORPTION COEFFICIENT

By the Method of Defined Geometry.—The shape of an aluminum absorption curve is very much dependent on the counting arrangement. It has been demonstrated that the closer an absorber is to the sample the larger the fraction of the radiation that is intercepted by the absorber, resulting in more total scattering. Thus the observation that more particles are scattered into the counter than are scattered out. Because of this scattering effect the absorbers should be placed as close to the window as possible. The arrangement used here, that of defined geometry, offers the advantage of placing the absorbers between the brass collimator and the window, reducing the fraction of radiation intercepted by the absorbers to that which is collimated.

A sample of I^{131} of known purity[7] was mounted for counting on Zapon film. Activity determinations were made individually and successively using at least six different thicknesses of aluminum chosen from the range giving 10 to 60 mg./cm.^{2} and a single absorber thicker than 250 mg./cm.^{2} The net beta counting rate was plotted on semilog paper as a function of the total absorber thickness. The slope of the line, i.e., the mass absorption coefficient, may be calculated

$$\mu = \frac{1}{\Delta t} \ln \frac{N_t}{N_t + \Delta_t} \qquad (14)$$

where N_t = corrected net beta counting rate with absorber of thickness t, and $N_{t+}\Delta_t$ = corrected net beta counting rate with absorber of thickness $t+\Delta t$ and where $t \geq 10$ mg./cm.^{2} and $\Delta t \geq 20$ mg./cm.^{2} Duplicate determinations made on the National Bureau of Standards sample showed a mass absorption coefficient of 0.0414 cm.^{2}/mg. ±1%. The theoretical value, calculated from Eq. 11, yielded 0.0413 cm.^{2}/mg.

By the Method of the Simulated I^{131} Reference Source.—The mass absorption coefficient (μ) is determined by removing the dural absorber and determining the counting rate with aluminum absorbers on shelf 1. The data is collected in the same manner as described previously and the net beta counting rate is plotted as a function of the total absorber thickness on semilog paper. A National Bureau of Standards sample analysis resulted in a value of 0.0320 cm.^{2}/mg. and two batches of I^{131} solution from the Oak Ridge National Laboratories gave 0.0324 and 0.0329 cm.^{2}/mg. It should be noted that this activity is mounted on copper and is counted on shelf 2, and that the spectrum of backscattered radiation is different from that of direct radiation.

RADIOCHEMICAL PURITY

Approximately one lambda of a solution of I^{131} was spotted one inch from the bottom of 18-inch strips of Whatman No. 1 filter paper (widths of 8, 10, and 20 mm.). The spots were air dried and a drop of carrier solution was then spotted at the same

[6] American Cyanamid & Chemical Corp., New York, N. Y.

[7] Obtained from the National Bureau of Standards, Washington, D. C.

site and dried. The carrier solution contained 1.66 mg. potassium iodide, 2.14 mg. potassium iodate, and 10 mg. sodium bicarbonate per ml. The chromatograms were developed over a period of about four hours by ascending chromatography using 75% methanol. The chromatogram was marked and dried in air. An automatic scanning and recording device (35) involving the use of a counting rate meter and recorder was used to represent graphically the activities at the iodide and iodate spots. A planimeter was used to determine the ratio of the iodide activity to the iodate activity. The R_f values obtained were 0.76 and 0.45 for iodide and iodate, respectively and were in correspondence with the R_f values determined by the conventional method of producing a color and visually estimating the center of the spots on the chromatogram itself.

The addition of carrier permitted chemical identification of the spots by spraying the paper with starch test solution, U. S. P. XIV, spraying with hydrogen peroxide solution, U. S. P. XIV, and finally spraying with ascorbic acid solution, 5%. The starch solution and hydrogen peroxide developed the iodide spot, while the starch solution and ascorbic acid solution developed the iodate spot.

DISCUSSION

As a result of this work, the following procedure of analysis is proposed for I^{131} which is to be used medicinally:

1. Primary calibration of the I^{131} activity by use of absolute beta counting with the collimated Geiger-Müller tube or the method of the simulated I^{131} reference source. Alternatively, the calibration may be carried out by the use of a currently available National Bureau of Standards calibrated sample of I^{131}.
2. Secondary calibration of the I^{131} activity by use of the electroscope.
3. Characterization of the beta radiation by the determination of the mass absorption coefficient.
4. Chemical identification of the sample by means of paper chromatography, with radio-assay of the spots to determine the per cent composition.

SUMMARY

Methods of assay of Sodium Radio-iodide (I^{131}) have been presented involving calibration of I^{131} using the electroscope, the method of absolute beta counting, and the method of the simulated I^{131} reference source. The details of each method have been described and discussed. The identification of the beta radiation through the use of the mass absorption coefficient is described. A test for the radiochemical purity involving chromatographic procedures is described.

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Synthesis and Antifungal Properties of 6-Halothymols*

By JOHN M. CROSS, CLARENCE A. DISCHER, and MICHAEL IANNARONE

The compounds 6-bromothymol and 6-iodothymol were prepared and their antifungal activities were compared with those of thymol and chlorothymol by *in vitro* test. Ten organisms, including six species of trichophyton, two species of microsporum, *candida albicans,* and *staphylococcus aureus,* were used in these tests. The tests indicate that at a level of 0.05% compound in propylene glycol solution, 6-iodothymol was the most effective agent, followed by 6-bromothymol, 6-chlorothymol, and thymol, in that order.

THYMOL AND CHLOROTHYMOL have been used for many years as antibacterial and antifungal agents (1, 2). Evidence has been accumulated to the effect that for antifungal activity of halogen compounds of phenols, bromine is superior to chlorine (3). It has also been reported that the order of antifungal activity in halogen compounds is as follows: iodine > bromine > chlorine (4, 5).

Since there is little information on the activity of halothymols other than chloro, it was thought advisable to prepare the compounds 6-bromo- and 6-iodothymol, and to compare their antifungal properties with those of thymol and 6-chlorothymol.

EXPERIMENTAL

Thymol.—Thymol N. F. was crystallized from petroleum ether (b. p. 30–75°). M. p. 50–51°.[1]

6-Chlorothymol.—Chlorothymol N. F. (6-chlorothymol) was crystallized from petroleum ether. M. p. 60–61°.

6-Bromothymol.—6-Bromothymol was prepared essentially by the method of Jost and Richter (6). Sixty grams (0.4 mole) of thymol was dissolved in 200 ml. of glacial acetic acid. Sixty-four grams of bromine (0.4 mole) was dissolved in 150 ml. of glacial acetic acid. The bromine solution was added slowly with stirring over a period of 20–30 minutes. The reaction mixture was allowed to stand overnight, and then poured into 1000 Gm. of crushed ice and water. A yellowish oil separated at the bottom of the beaker. Sodium bicarbonate was then added to neutralize the acid. The oil solidified as the *p*H approached 8. The solid was separated and dried over $CaCl_2$ for several days. The dry, crude yield was 53 Gm. (58% of theory). Crystallization of the product from petroleum ether yielded white crystals. M. p. 55–55.5°.

Anal.—Calcd. for $C_{10}H_{13}BrO$: C, 52.42; H, 5.72; Br, 34.88. Found: C, 52.67; H, 5.94; Br, 34.61.

TABLE I.—INHIBITION (DIAM. IN MM.) AGAINST THREE ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism No. 1[b] S	Organism No. 1[b] SS[c]	Organism No. 2[c] S	Organism No. 2[c] SS	Organism No. 3[d] S	Organism No. 3[d] SS
Thymol	1.00	8.00	8.00	2.00	2.00	...	...
Thymol	0.10	0.00	0.00	0.00	0.00	0.00	0.00
Thymol	0.05	0.00	0.00	0.00	0.00	...	...
6-Chlorothymol	1.00	25.00	25.00	13.00	10.00	...	...
6-Chlorothymol	0.10	6.00	4.00	2.00	2.00	3.50	1.50
6-Chlorothymol	0.05	...	...	0.00	0.00	...	...
6-Bromothymol	1.00	22.00	22.00	13.00	11.00	...	...
6-Bromothymol	0.10	14.00	14.00	5.00	5.00	...	...
6-Bromothymol	0.05	5.00	0.00	3.00	3.00	3.00	1.50
6-Iodothymol	1.00	17.00	17.00	9.00	9.00	...	...
6-Iodothymol	0.10	11.00	8.00	3.00	3.00	...	...
6-Iodothymol	0.05	9.00	9.00	3.00	3.00	4.50	1.50
2,6-Dibromothymol	1.00	...	...	...	...	...	...
2,6-Dibromothymol	0.10	0.00	0.00	...	...	...	...
2,6-Dibromothymol	0.05	0.00	0.00	...	...	...	...
Propylene glycol[f]	100.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each result is an average of three plates for each percentage tested in the presence of 5.0% horse serum and after 14 days incubation.
[b] *Trichophyton mentagrophytes* (598 B). [c] *Candida albicans.* [d] *Microsporum canis* (*Stilliansi*) (234).
[e] S = Surface, SS = Subsurface.
[f] Sterile propylene glycol was used as a control and solvent for all of the halothymols.

2,6-Dibromothymol.—This compound was prepared according to the above process by doubling the quantity of bromine used (6). A light yellow oil was obtained. Since the oil showed little antifungal activity it was not further characterized.

* Received May 5, 1955, from the Research Laboratories of Rutgers University College of Pharmacy, Newark 4, N. J.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.
[1] All melting points reported were determined by means of the Fisher-Johns apparatus, and are uncorrected.

TABLE II.—INHIBITION (DIAM. IN MM.) AGAINST THREE ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism No. 1[b] S	Organism No. 1[b] SS[c]	Organism No. 2[c] S	Organism No. 2[c] SS	Organism No. 3[d] S	Organism No. 3[d] SS
Thymol	0.10	0.00	0.00	0.00	0.00	12.50	12.00
6-Chlorothymol	0.10	8.00	8.00	12.00	12.00	NR[f]	NR
6-Chlorothymol	0.05	0.50	0.50	...	...	...	...
6-Bromothymol	0.05	13.50	11.50	7.50	5.00	NR	NR
6-Iodothymol	0.05	14.50	11.50	13.50	9.50	12.00	12.00
Propylene glycol[g]	100.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each result is an average of two plates for each percentage tested after 14 days incubation.
[b] *Trichophyton purpureum* (Emmons 661). [c] *Trichophyton interdigitale*. [d] *Trichophyton interdigitale* (Emmons 640).
[e] S = Surface, SS = Subsurface.
[f] NR = No results.
[g] Sterile propylene glycol was used as a control and solvent for all of the halothymols.

TABLE III.—INHIBITION AGAINST THREE ORGANISMS BY 6-HALOTHYMOLS[a]

Thymol Compound	%	Organism No. 1[b] S	Organism No. 1[b] SS[c]	Organism No. 2[c] S	Organism No. 2[c] SS	Organism No. 3[d] S	Organism No. 3[d] SS
Thymol	0.10	0.00	0.00	1.00	1.00	0.00	0.00
6-Chlorothymol	0.10	8.50	8.00	NR[f]	NR	NR	NR
6-Chlorothymol	0.05	2.00	2.50	...	...	...	...
6-Bromothymol	0.05	14.50	14.00	11.00	11.00	5.00	5.00
6-Iodothymol	0.05	15.50	15.50	14.50	14.50	12.00	11.00
Propylene glycol[g]	100.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each result is an average of two plates for each percentage tested after 14 days incubation.
[b] *Microsporum felineum*. [c] *Trichophyton rubrum*. [d] *Trichophyton gypseum*.
[e] S = Surface, SS = Subsurface.
[f] NR = No results.
[g] Sterile propylene glycol was used as a control and solvent for all of the halothymols.

TABLE IV.—INHIBITION (DIAM. IN MM.) AGAINST STAPHYLOCOCCUS AUREUS 6-HALOTHYMOLS[a]

Thymol Compound	%	*Staph. aur.* S	*Staph. aur.* SS[b]
Thymol	0.10	0.00	0.00
6-Chlorothymol	0.10	6.00	6.00
6-Chlorothymol	0.05	2.00	2.00
6-Bromothymol	0.05	3.50	3.50
6-Iodothymol	0.05	4.00	4.00
Propylene glycol[c]	100.00	0.00	0.00

[a] Each result is an average of two plates for each percentage tested after 48 hours incubation.
[b] S = Surface, SS = Subsurface.
[c] Sterile propylene glycol was used as a control and solvent for all of the halothymols.

6-Iodothymol.—6-Iodothymol was prepared by the method of Woolett and Everett (7), and also by the following method which was found to be more convenient: 30 Gm. of thymol (0.2 mole) and 34 Gm. of KOH (0.6 mole) were dissolved in 100 ml. of methanol. In another beaker 50.8 Gm. (0.2 mole) of iodine was dissolved in 500 ml. of methanol. The thymol solution was cooled to 0–5° and stirred while the iodine solution was added slowly over a period of 20–30 minutes. The reaction mixture was then poured into 1000 Gm. of crushed ice and water. Dilute HCl was added to bring the solution to *p*H 6–7. A white crystalline precipitate formed, which was separated, washed with water, and dried overnight on filter paper. The crude yield was 49.7 Gm. (90% of theory).

Crystallization of the product from petroleum ether yielded 36 Gm. (65%) of white crystals and 13 Gm. of a yellow oil from which no further crystals could be obtained. M. p. 68–69°.

Anal.—Calcd. for $C_{10}H_{13}IO$: C, 43.49; H, 4.75; I, 45.96. Found: C, 43.83; H, 5.14; I, 45.90.

Antifungal Activity.—In order to evaluate the antifungal properties of the 6-halothymols, *in vitro* studies were performed to compare their effectiveness against a number of dermatophytes chiefly of the genera *Trichophyton* and *Microsporon*, and against *Candida albicans*. As there is such a wide variation in the testing of these compounds *in vitro*, the method decided upon was that of the F. D. A. serum agar cup plate method as described in Circular #198 (8). Since serum agar cup plates do not differentiate between fungistatic and fungicidal action, fungistatic action was determined by the standard technic of measuring surface and subsurface zones of inhibition.

A preliminary investigation indicated that all of the thymol derivatives were active in concentrations of 5% or more. It was in the range of 0.05% to 1.0% that significant differences in activity were noticed. Consequently this latter range was used for purposes of comparison. Tables I, II, III, and IV summarize the results of antifungal activity of thymol, 6-chlorothymol, 6-bromothymol and 6-iodothymol against ten organisms.

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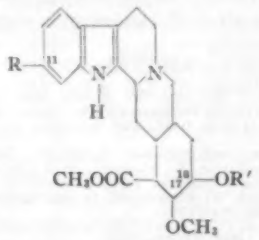
Raunescine and Isoraunescine from *Rauwolfia canescens* L.*

By NORMAN HOSANSKY and ERIC SMITH

Two new ester alkaloids have been isolated from *Rauwolfia canescens* L. Their isolation, physical properties, and probable structure are reported.

THREE ALKALOIDS possessing hypotensive and sedative activity have been isolated from Rauwolfia species: reserpine (1), canescine (2), and rescinnamine (3). The basic structure of these alkaloids has been elucidated. They correspond to Formula I.



(I)

R in canescine is hydrogen; in reserpine and rescinnamine R represents the methoxyl group. R′ in canescine and reserpine represents the 3,4,5-trimethoxybenzoyl group, whereas in rescinnamine it represents the 3,4,5-trimethoxycinnamoyl group. The biological activity of all three compounds seems to depend on the esterified hydroxyl group at the C^{18} position (4).

We have succeeded in isolating from the weakly basic alkaloid fraction of *R. canescens* two additional alkaloids esterified with 3,4,5-trimethoxybenzoic acid for which we propose the names *raunescine* and *isoraunescine*.

Raunescine was isolated as its monohydrate in hexagonal prisms which soften and melt (unsharp) between 160° and 170°. Its specific rotation is −74° (c = 1 in chloroform). Isoraunescine crystallizes in rods, has a melting point 241–242.5° and a specific rotation of −70° (c = 1 in chloroform). Both alkaloids on alkaline hydrolysis give the equivalent of 1 mole of trimethoxybenzoic acid.

The analytical data and the ultraviolet and infrared spectra suggest that raunescine and isoraunescine are very similar in structure to canescine and may differ from it only in that they possess at the C^{17} carbon a hydroxyl instead of the methoxyl group found in canescine.

* Received August 30, 1955, from the Research Division of S. B. Penick & Co., Jersey City, N. J.

EXPERIMENTAL

Isolation.—The weakly basic alkaloid fraction (105 Gm.) from *R. canescens*, from which reserpine and canescine had been removed, was dissolved in 1 L. of 5 *N* acetic acid and the solution was extracted with ether for the removal of color. The solution was then alkalinized with potassium carbonate and extracted with ether. Raunescine and isoraunescine were not soluble in this solvent but were later extracted with chloroform. The chloroform extract was concentrated to give 55 Gm. of amorphous material. This residue was redissolved in 200 ml. of chloroform and put on a column of 1500 Gm. of silica gel. The column was developed first with chloroform (3 L.) and then with chloroform containing 2% methyl alcohol (15 L.). The eluates were collected in 500-ml. fractions. The chloroform–2% methyl alcohol eluates contained in the first five fractions reserpine and canescine (2 Gm.). The weight of subsequent fractions, after first diminishing, increased and the eighth to thirteenth fractions gave on concentration about 20 Gm. of residue which, when treated with 90% methyl alcohol, crystallized to give 6 Gm. crude raunescine. Later fractions diminished in weight; the column was therefore developed with chloroform—5% methyl alcohol (10 L.) and the fractions collected as before. These fractions gave, after concentration and treatment with aqueous 90% methyl alcohol, 3 Gm. crude isoraunescine.

Purification and Properties of Raunescine.—The base was recrystallized to constant rotation from 90% methyl alcohol.

Anal.[1]—Calcd. for $C_{31}H_{38}N_2O_8.H_2O$ (582.6): C, 63.90; 6.57; N, 4.81; 4 OCH_3, 21.31. Found: C, 63.96, 64.15; H, 6.30, 6.43; N, 5.08, 4.85; OCH_3, 20.67. Molecular weight by anhydrous titration with 0.1 *N* perchloric acid, 584.5.

Raunescine gave a difficultly soluble nitrate with a melting point of 223–225°, $[\alpha]_D$ −80° (c = 1 in 5 *N* acetic acid).

Anal.—Calcd. for $C_{31}H_{38}N_2O_8.HNO_3$: C, 59.32; H, 5.94; N, 6.70; 4 OCH_3, 19.78. Found: C, 60.05; H, 6.10; N, 6.38; OCH_3, 21.94.

Hydrolysis with sodium hydroxide in aqueous methyl alcohol gave 1 mole of 3,4,5-trimethoxybenzoic acid, m. p. 169–170° with no melting point depression on admixture with an authentic sample. The basic part of the molecule has not yet been brought to crystallization.

When treated with acetic anhydride and pyridine, raunescine gave a monoacetate with a m. p. of 159–162° and $[\alpha]_D$ −157° (c = 1 in chloroform).

Anal.—Calcd. for $C_{33}H_{40}N_2O_9.H_2O$: C, 63.43; H, 6.45; N, 4.49. Found: C, 63.72; H, 6.33; N, 4.45.

The acetyl compound formed a crystalline nitrate

[1] Analyses by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.

with a m. p. 233–235°, $[\alpha]_D$ −129° (c = 1 in 5 *N* acetic acid).

Anal.—Calcd. for $C_{32}H_{38}N_2O_9.HNO_3$: C, 59.19; H, 5.87; N, 6.28. Found: C, 59.39; H, 6.43; N, 6.29.

Purification and Properties of Isoraunescine.—The crude base was recrystallized to constant melting point from 80% methyl alcohol.

Anal.—Calcd. for $C_{31}H_{36}N_2O_8$ (564.6): C, 65.93; H, 6.43; N, 4.96; 4 OCH_3, 22.67. Found: C, 65.67; H, 6.50; N, 4.88; OCH_3, 21.31. Molecular weight by anhydrous titration with 0.1 *N* perchloric acid, 569.

From alcoholic solution with nitric acid a crystalline nitrate was obtained with m. p. 254–255°, $[\alpha]_D$ −80° (c = 1 in 5 *N* acetic acid).

Anal.—Calcd. for $C_{31}H_{36}N_2O_8.HNO_3$: C, 59.32; H, 5.94; N, 6.70; 4 OCH_3, 19.78. Found: C, 59.87; H, 6.02; N, 6.59; OCH_3, 19.67.

On treatment with acetic anhydride and pyridine a crystalline acetyl compound was isolated as the nitrate with a m. p. 242–245°, $[\alpha]_D$ −68° (c = 1 in 5 *N* acetic acid).

Anal.—Calcd. for $C_{33}H_{38}N_2O_9 \cdot HNO_3 \cdot C_2H_5OH$: C, 58.73; H, 6.34; N, 5.87. Found: C, 58.76; H, 6.28; N, 6.13.

Ultraviolet Spectra.—The ultraviolet absorption spectra of both compounds, as determined in U. S. P. alcohol, were extremely similar. Raunescine had a maximum at 218 mμ (log ε 4.77) and at 271 mμ (log ε 4.26), and a minimum at 243 mμ (log ε 3.82). Isoraunescine had a maximum at 218 mμ (log ε 4.76) and at 270 mμ (log ε 4.24), and a minimum at 242 mμ (log ε 3.85). The absorption curves are almost identical with that of canescine.

Infrared Spectra.—The infrared absorption spectra, determined as a Nujol® mull using a Perkin-Elmer Model 21 instrument, showed for raunescine bands at 2.82 and 2.87 μ, and a doublet at 5.68 and 5.82 μ. Isoraunescine had bands at 2.81 and 2.87 μ, and showed only a single band at 5.79 μ. Neither compound showed an absorption band at 6.18 μ. This band, which is also missing in canescine, is associated with the 11-methoxyl group.

SUMMARY

Two new isomeric alkaloids, both giving 3,4,5-trimethoxybenzoic acid on hydrolysis, have been isolated from *Rauwolfia canescens* L. From their chemical and physical properties a structural relationship to the known pharmacologically active alkaloid, canescine, is suggested.

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The Chromatographic Analysis of Reserpine Preparations*

By DANIEL BANES, JONAS CAROL, and JACOB WOLFF

Methods are presented for the analysis of reserpine preparations by chromatography on paper and on liquid-liquid partition columns. The former is used for the qualitative examination of samples and the latter for the quantitative assay of reserpine contents. The system of paper chromatography permits the identification of reserpine and the detection of the closely related concomitants deserpidine, rescinnamine, ajmaline, ajmalicine, and serpentine. The partition column can be used for milligram quantities of samples.

AN EARLIER PUBLICATION from this laboratory (1) described procedures for extracting the reserpine fraction from drugs, and established criteria for the purity of the extracts. McMullen and co-workers (2) recently have reported a chromatographic procedure for the analysis of reserpine in various commercial preparations. Their procedure is designed to separate reserpine from serpentine, ajmaline, ajmalicine, and certain decomposition products. Commercial preparations of reserpine may also contain its congeners deserpidine (canescine, recanescine) and rescinnamine (3–5). The system proposed here for paper chromatography isolates reserpine and permits the detection and identification of closely related concomitants. The partition column accommodates reserpine in milligram quantities, and the eluate is suitable for direct spectrophotometric analysis.

* Received September 5, 1955, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

EXPERIMENTAL

Column Chromatography

Reagents.—*Solvents.*—Chloroform, alcohol, and isooctane of U. S. P. grade. Shake the isooctane with several portions of concentrated sulfuric acid, wash with water, and distill in all-glass apparatus.

Solutions.—(*a*) Citric acid solution. Dissolve 10.50 Gm. of U. S. P. citric acid in water, and dilute to exactly 500 ml. (*b*) Immobile solvent. Mix 50.0 ml. of citric acid solution with 20.0 ml. of alcohol, and cool. (*c*) Eluent. Shake exactly 100 ml. of chloroform with exactly 200 ml. of isooctane, 100 ml. of water, and 40 ml. of alcohol for twenty minutes. Discard the aqueous layer and filter the upper layer rapidly through paper. Prepare the chromatographic solutions on the day of use. (*d*) Sodium bicarbonate solution, 2% w/v, free from sodium carbonate.

Standard Reserpine Solution 0.5 mg./ml.—Dissolve 50.0 mg. of crystalline reserpine in about 40 ml. of hot alcohol. Dilute with 50 ml. of alcohol, cool, dilute to exactly 100 ml., and mix. Stored in the dark in a tightly stoppered brown glass bottle, the standard is stable for several weeks.

Diatomaceous Silica Adsorbant.—Johns-Manville Celite® 545. *Apparatus.*—Chromatographic tube. A 400-mm. length of glass tubing with an inner diameter of 22–23 mm., fused to a 50-mm. length of outlet tubing (inner diameter about 6 mm.), and fitted with a packing rod and reservoir (6).

Preparation of the Column.—Place a small wad of purified cotton in the bottom of the chromatographic tube. Prepare the wash layer (6) by mixing 1 ml. of sodium bicarbonate solution and 0.4 ml. of alcohol with 1.5 Gm. of adsorbant, transfer to the tube, and press down with the packing rod. Cover with about 0.5 Gm. of adsorbant. Prepare a uniform trap layer by thoroughly mixing 10.0 ml. of immobile solvent with 10 Gm. of adsorbant, and transfer to the tube through a powder funnel in two or three portions without loss, pressing with the rod after each addition. Rinse the beaker with about 0.5 Gm. of Celite® and transfer to the tube. The height of the trap layer should be 10–12 cm.

Analysis of the Standard.—Protect reserpine samples from direct or strong light at all times, and complete analyses promptly. Transfer 4.0 ml. of the reserpine standard solution to a 400-ml. beaker and add about 12 ml. of the eluent. Thoroughly mix 10 ml. of 2% sodium bicarbonate solution with 15 Gm. of adsorbant, and add to the prepared reserpine mixture, stirring to homogeneity. Transfer the sample layer to the tube quantitatively, as previously described (6). Add eluent to the tube, and permit it to percolate through the column, receiving the forerun in a graduated cylinder. Adjust the level of the liquid above the column to yield a flow-rate of 2–4 ml. per minute. The volume of the forerun, in ml. is given by the formula, $t + 18$, where t is the temperature in degrees Centigrade. Replace the cylinder with a 100-ml. volumetric flask, and collect about 99 ml. of eluate. Rinse the stem with a few drops of eluent solution, dilute the reserpine fraction to exactly 100 ml. with the solvent, and mix. Determine the ultraviolet absorption spectrum of the prepared standard solution relative to the eluent as blank between 245 and 330 mμ. The spectrum should show maxima at 268 and 295 mμ, and minima near 248 and 288 mμ. The ratio of the maximum absorbances (A_{268}/A_{295}) should be 1.73 ± 0.05.

Powdered Reserpine.—Dissolve an accurately weighed 20–30 mg. sample of powdered reserpine in 10 ml. of chloroform, and dilute to exactly 50 ml. with alcohol. Immediately transfer a 5-ml. aliquot of the solution to a 400-ml. beaker, mix with 2 ml. of isooctane and 10 ml. of eluent, and complete the assay as directed under *Analysis of Standard*, beginning with the words "Thoroughly mix 10 ml. of 2% sodium bicarbonate solution...." Calculate the reserpine content of the sample by comparison of the sample eluate absorbance at 268 mμ with that of the standard eluate.

Tablets.—Transfer a powdered sample of tablets, equivalent to 1.5–3.0 mg. reserpine, to a 400-ml. beaker and mix with 4 ml. of alcohol. Cover with a watch glass, immerse in a water bath slightly above the level of the mixture, and heat at 55 ± 5° for fifteen minutes with frequent swirling. Cool, mix with 5 ml. of chloroform, and immerse in the bath for one minute. Allow to cool for fifteen minutes, with frequent swirling. Add 10 ml. of isooctane and the adsorbant-bicarbonate mixture, and analyze as directed above.

Injections.—Transfer an aliquot containing 1.5–3.0 mg. reserpine to a 400-ml. beaker, and mix with 4 ml. of alcohol. Add 12 ml. of eluent and the adsorbant-bicarbonate mixture, and proceed as directed above.

Paper Chromatography

Reagents.—*Solvents.*—Acetone (Mallinckrodt A. R.); ammonium hydroxide reagent grade; cyclohexanol (Eastman Kodak); redistilled benzene and isooctane (see solvents under "Column Chromatography" for procedure) and redistilled formamide. Shake formamide (Fisher Scientific Co.) with anhydrous K_2CO_3, filter and distill under vacuum using all-glass apparatus (b. p. 101° at 12 mm. Hg). Store in desiccator over H_2SO_4 until free of NH_3.

Solutions. (*a*) Immobile solvent. Dilute 30 ml. of formamide to 100 ml. with acetone. (*b*) Mobile solvent. Shake 100 ml. isooctane, 50 ml. benzene and 5 ml. formamide for five minutes. Discard the lower layer, add 2 ml. cyclohexane to the upper layer and filter rapidly through paper. (*c*) Reference solutions. Prepare alcoholic solutions of reserpine, rescinnamine, deserpidine ajmalinol, ajmalicine, and serpentine[1] to contain 200–500 micrograms per ml.

Apparatus.—8 x 8-inch sheets of Whatman No. 1 filter paper. A rectangular metal tank 8½ x 3 x 8½ inches with glass lid and paper hangers suitable for ascending chromatography. The tank is equipped with a blotting paper liner, dipping into the mobile solvent, and a glass tray for NH_4OH to saturate the atmosphere with NH_3.

Procedure.—Place *ca.* 10 ml. of NH_4OH in the glass tray. Transfer the mobile solvent (*b*) to the bottom of the tank outside the glass tray. Keep covered with the glass lid to reduce evaporation losses.

Mark points for application of solutions on a line one inch from the bottom of a sheet of filter paper.

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[1] These alkaloids were kindly provided by the Riker Laboratories, Los Angeles, Calif.

Ten spots can be placed on a sheet quite readily. Immerse the paper in immobile solvent (*a*) and completely remove excess solution by blotting between paper toweling. For optimal results the formamide must be spread uniformly over the paper. Spot with 5–10 λ portions of sample and reference solutions and allow the alcohol solvent to dry.

Suspend the paper into the tank so that the bottom dips into the mobile solvent. Seal the glass lid with scotch tape and cover with an opaque object to minimize photodecomposition.

In about one hour, when the mobile solvent has risen approximately $^7/_8$ of the height of the paper, remove the chromatogram and dry at 90° in a current of air. Exposure of the paper to HCl fumes for several minutes at this point usually increases the fluorescence of deserpidine. Examine the chromatogram under an ultraviolet lamp and encircle the fluorescent spots in pencil.

DISCUSSION

The method proposed for the isolation of reserpine by paper chromatography was based upon existing methods (7–9) and extensive experimentation[1] with immiscible solvent systems, temperature and *p*H effects, and the diffusion properties of the Rauwolfia alkaloids.

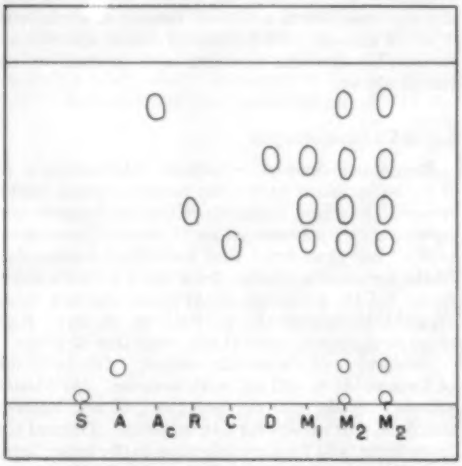

Fig. 1.—Chromatogram showing separation of six Rauwolfia alkaloids. S = Serpentine, A = Ajmaline, A_c = Ajmalicine, R = Reserpine, C = Rescinnamine, D = Deserpidine, M = Mixture of R, C, and D. M_2 = Mixture of all six.

Figure 1 shows the separation of the six alkaloids studied. As can be noted, each is completely separated from the others. The fluorescent spots of the alkaloids vary considerably in intensity and hue. Those of reserpine, rescinnamine, and ajmalicine exhibit about the same intensity and a similar green color. Deserpidine fluoresces a somewhat weaker blue-green. Serpentine gives an intense blue spot and ajmaline a weak blue that appears only after a day or more. Figure 2 shows the analysis of crystalline reserpine from a number of manufacturers' lots. Several samples exhibited a blue-fluorescing spot in a position corresponding to that of deserpidine, but no other alkaloids were detected. In addition, most of the samples showed one or more very faint spots including a yellow-fluorescing one, which remained near the starting line. These were omitted from Figure 1 to avoid confusion with the spots due to ajmaline and serpentine. The sample with the smallest quantities of extraneous substances was adopted as the reserpine standard for column chromatography.

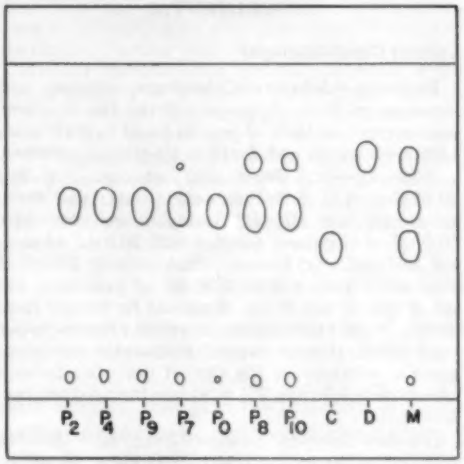

Fig. 2.—Chromatogram of crystalline reserpine from various sources. P_0 refers to powders listed in Table I. C = Rescinnamine, D = Deserpidine, and M = Mixture of R, C, and D.

In order to test the sensitivity of the method, a series of mixtures of reserpine and rescinnamine containing 500 μg. per ml. of total alkaloids were chromatographed. As little as 1% of either could be detected in the presence of the other. The R_f values appeared to be independent of concentration.

Solvents for column chromatography eventually were chosen considering the solubility and stability of reserpine, and convenience in preparing samples for analysis. Excessive alcohol in the sample layer was found to cause slight trailing, but proportions of alcohol as low as 3 parts, to 10 parts of aqueous solution in the sample layer, had no effect on the elution rate of reserpine. The formula $t + 18$ for the forerun volume was based upon experiments conducted between 18 and 35°. More than 10 mg. of reserpine could be analyzed on a single column, and the absorbances of a series of sample eluates obeyed Beer's law at both 268 and 295 mμ. Although reserpine decomposed slowly in these solutions, the absorbances at 268 mμ remained constant for a week.

Chromatographic analysis of standard reserpine yielded an eluate with a peak absorbance equal to 99.3% of the value obtained for an untreated sample in eluent solution. The actual readings for a series of standard replicates over a period of four weeks ranged from 0.528 to 0.541 for one analyst, and

[1] We are deeply grateful to Lloyd C. Mitchell and to Joseph Levine, of the U. S. Food and Drug Administration, Washington, D. C., for their valuable suggestions on chromatographic procedures.

TABLE I.—RESERPINE ASSAYS BY COLUMN CHROMATOGRAPHY

Sample	Description	Reserpine Found — Analyst I	Analyst II
Controls			
C-1	4.0 ml. standard plus 4 Gm. potato starch plus 0.4 Gm. lactose	2.04 mg.	
C-2	No reserpine; 4 Gm. starch plus 0.4 Gm. lactose	0.03 mg.	
C-3	4.0 ml. standard plus 16 mg. mixed protoveratrines	2.00 mg.	
C-4	0.096% reserpine in tablet mixture containing starch, talc, lactose, etc.	0.097%	
		0.096	0.099%
		0.098	
Powders		Per cent	Per cent
P-0	White crystals, source unknown	100.0	...
P-1	White crystals, *micrantha*	...	98.8[a]
		...	100.0
P-2	White crystals, source unknown	97.8	100.0
P-3	White crystals, *heterophylla*	98.6	...
P-4	White crystals, *vomitoria*	95.3	95.6
			97.5
P-5	White crystals, *heterophylla*	...	97.7
P-6	Pale yellow crystals, *serpentina*	96.4	96.8
P-7	White crystals, *canescens*	98.6[b]	...
P-8	White crystals, source unknown	...	98.1[b]
P-9	White crystals, source unknown	92.4	92.8
P-10	Yellow crystals, source unknown	82.7	84.8
Tablets		mg.	mg.
T-1	Declared 0.25 mg./tablet	0.233	...
T-2	Declared 0.1 mg./tablet	0.098	0.096
T-3	Declared 1.0 mg./tablet	0.954	0.936
T-4	Declared 0.25 mg./tablet	0.244	...
T-5	Declared 0.1 mg./tablet	...	0.100
T-6	Declared 0.25 mg./tablet	0.230	...
T-7	Declared 0.1 mg./tablet	0.098	0.097
T-8	Declared 0.1 mg./tablet	...	0.095
T-9	Declared 0.25 mg./tablet	0.233	0.244
T-10	Declared 0.25 mg./tablet	...	0.219
T-11	Declared 0.25 mg./tablet	0.227	0.224
T-12	Declared 1.0 mg./tablet	...	0.926
T-13	Declared 0.1 mg./tablet	0.086	0.089
T-14	Declared 0.1 mg./tablet	...	0.097
T-15	Declared 0.1 mg./tablet	...	0.091
T-16	Declared 0.25 mg./tablet	...	0.219
T-17	Declared 0.25 mg./tablet	0.214	0.216
T-18	Declared 0.1 mg./tablet	...	0.096
T-19	Declared 0.1 mg./tablet	...	0.111
T-20	Declared 0.08 mg./tablet Also contained protoveratrines	0.062	...
T-21	Declared 0.1 mg./tablet Also contained hyoscine, atropine, hyoscyamine	0.097	...
T-22	Declared 0.1 mg./tablet Also contained rutin	Method failed	
T-23	Declared 0.1 mg./tablet Also contained quercetin		
Injections		mg.	mg.
I-1	Declared, 2.5 mg./ml.	2.41	...
		2.39	2.40
I-2	Declared, 2.5 mg./ml.	2.42	2.44
I-3	Declared, 2.5 mg./ml.	2.26	2.24
		...	2.23

[a] Sample weighed 11.1 mg. [b] Forerun cuts contained large concentrations of deserpidine.

0.512 to 0.533 for another. It is probably unnecessary to analyze a standard daily.

Several control samples were prepared to test the accuracy of the method on simulated tablet mixtures (C-1 to C-4) (Table I). Recoveries were considered satisfactory. Check values obtained in the analysis of simulated tablet mixture C-4, and in succeeding samples, are evidence for a high degree of reproducibility.

Samples containing deserpidine also were prepared, and the behavior of this alkaloid in the standard chromatographic system was studied. Deserpidine appeared in the forerun, and trailed into the reserpine fraction. Spectrophotometric analysis of these forerun fractions was found useful for detecting deserpidine in significant amounts, and was applied to all assays. A small portion of the eluate following the reserpine fraction also was examined routinely as a test for complete elution.

Several series of analyses were made on reserpine

powders, tablets and injectables by the proposed method and are reported in Table I. The samples of "P" series were previously examined by paper chromatography. Sample P-0 was an independent portion of standard reserpine treated as a crystalline powder. The assay for this sample, and for P-1, was 100%. Crystalline powders 3–7 had an apparent reserpine content of 95% or more. The forerun cuts of P-7, allegedly derived from *Rauwolfia canescens*, contained deserpidine. In P-8, the eluate exhibited no absorbance maximum at 295 mμ, and the forerun cuts showed large concentrations of deserpidine.[3] The assay value of P-9 was 92–93% reserpine, and only traces of deserpidine were detected. Examination of the eluted column under ultraviolet light revealed a large yellow-fluorescing band near the top of the trap layer. This polarophilic material was eluted with 80 ml. of chloroform. The collute[4] in chloroform solution possessed an absorption maximum at 268 mμ, corresponding to 10% of the original sample, when calculated as reserpine. Less than 1% of this constituent occurred in the standard, while P-10 contained it, as well as deserpidine, in large proportions. Correlation between these findings and the chromatograms developed on paper were excellent. The assay for reserpine in P-10 by the chromatographic method was 83–85%, while the vanillin colorimetric method (1) yielded a value slightly more than 80%.

In the series of tablet samples representing 23 different manufacturers' lots, every assayable reserpine fraction (Table I) exhibited a normal ultraviolet absorption spectrum. Sample T-20 compounded with veratrum alkaloids, contained only 78% of the declared quantity of reserpine, and large proportions of the polarophilic yellow-fluorescing substance. Previous analyses by the colorimetric and hydrolytic procedures yielded similar results (1). The chromatographic method failed when applied to tablets containing quercetin or rutin. The flavones continuously eluted during analysis, and interfered with direct spectrophotometric measurements. For similar reasons, the colorimetric-hydrolytic method following extraction was found preferable for the analysis of elixirs.

The injection samples examined were labeled to contain 2.5 mg. of reserpine per ml. of solution, and 1.0 ml aliquots were analyzed. (With lower concentrations, the acidic solution should be neutralized with saturated sodium bicarbonate solution, and smaller volumes of the 2% solution should be mixed with the 15 Gm. charge of adsorbant to yield a total aqueous component of 10 ml. in the sample layer.) Injection Sample I-3 contained reserpine equivalent to only 89–90% of the declared quantity, and a correspondingly high polarophilic fraction. The nature of this fraction, which probably includes degradation products derived from reserpine, is under investigation in this laboratory.

[3] Spectrophotometric analysis of consecutive collute fractions (see footnote 4) demonstrated that deserpidine comprised about 17% of Sample P-8. Details of this analytical procedure will be published elsewhere.

[4] We propose the term "collute" to designate the substance or substances removed from a chromatographic system together with the eluent to form the eluate (L. *Col*—with, together and *luere*—to wash).

SUMMARY

Chromatographic methods have been presented for the analysis of commercial reserpine preparations. These methods separate reserpine from closely-related concomitants, and permit its assay by ultraviolet spectrophotometry.

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A Note on the Polarographic Determination of Ionic Copper Present in the Commercially Available Copper Chlorophyllins*

By WALTER L. WUGGATZER† and JOHN E. CHRISTIAN

In recent publications (1, 2) we reported on the polarographic behavior of a series of commercially available chlorophyllins. The reduction waves, at $E^1/_2$ of −0.26 and −0.53 volt *vs.* the saturated calomel electrode (S. C. E.), of the copper chlorophyllins in ammoniacal solution were believed caused by the ionic copper not completely removed after the manufacture of the chlorophyllins.

Further study has proved this speculation to be correct. The addition of copper sulfate to several chlorophyllins not containing copper resulted in the appearance of the copper waves when the ammoniacal solutions were electrolyzed at the dropping mercury electrode. Additional proof was obtained from studies on a series of copper chlorophyllins in which the amount of ionic copper present had previously been determined by the manufacturers. Upon electrolysis of these chlorophyllins at the dropping mercury electrode, it was found that those chlorophyllins said to contain ionic copper did give the two reduction waves believed caused by copper, and those said not to contain ionic copper did not give the two waves.

The above findings led to the development of a polarographic method for the determination of ionic copper present in copper chlorophyllins. The, method is as follows: the capillary characteristics the instrument used, and the conditions maintained were the same as previously presented (1).

Known amounts of copper sulfate, previously dried to constant weight, were dissolved in 1.5 *M* ammonia-ammonium chloride solution and the solutions electrolyzed at the dropping mercury electrode. The method devised by Kolthoff and Lingane (3) for the determination of copper was used. A graph was constructed by plotting the corrected diffusion currents (corrected by subtracting the residual current of the ammoniacal solution from the total diffusion current of the two copper waves) against copper concentrations. A linear relationship was obtained.

Known concentrations of the copper chlorophyllins in 1.5 *M* ammonia-ammonium chloride solution were then electrolyzed at the dropping mercury electrode. The concentration of ionic copper present was determined from the graph constructed for pure copper sulfate using the corrected diffusion current obtained (corrected by subtracting the residual current obtained for ionic copper-free copper chlorophyllins from the total diffusion current of the two copper waves). Concentrations of ionic copper present in commercially available copper chlorophyllins, as determined by this method, ranged from 0.17 to 1.35 per cent.

The present methods used by manufacturers of copper chlorophyllins to determine the amount of ionic copper present have not been revealed, however, the methods used have been stated to be quite tedious and subject to considerable error. The polarographic method would seem to be an easier and more accurate procedure.

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(1) Wuggatzer, W. L., and Christian, J. E., *Science*, **118**, 444(1953).

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Book Notices

Advances in Enzymology and Related Subjects of Biochemistry. Vol. 16. Edited by F. F. Nord. Interscience Publishers, Inc., New York, 1955. x + 584 pp. 16.5 x 23.5 cm. Price $11.

The chapters in this volume of the series, aside from the contribution on coenzyme A by J. Baddiley of England, represent the efforts of American investigators. The previous volume (XV), noted in This Journal, **43**, 639(1954), had a more foreign flavor. The present text includes chapters on: The Structure of Coenzyme A; Coagulation of the Blood; Comparative Biochemistry of the Phenolase Complex; Transamination; Intermediates in Amino Acid Biosynthesis; Structural and Functional Aspects of Myosin; Beta-Glucuronidase; and The Chemistry of the Cell Nucleus. Adding to the reference value of the book are the extensive bibliographies, running to 538 references on the phenolase complex and to more than 300 on other subjects, the author and subject indexes for Vol. XV, and the cumulative author and subject indexes for Vols. I to XVI.

Technique of Organic Chemistry. Vol. 7. Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., New York, 1955. vii + 552 pp. 16 x 24.75 cm. Price $8.50.

This book, which is the seventh in a series of nine volumes, presents extensive data on the physical properties and constants of organic solvents, much of the data being tabulated. The solvents are classified according to chemical principles and are numbered for ready reference throughout the text. Inclusion of recent material in this reference volume is probably the reason Vol. VII in this series was delayed beyond the publication of Vol. VIII which was reviewed in THIS JOURNAL, **42**, 507(1953).

The arrangement and text follows the style established in the first edition by A. Weissberger and E. S. Proskauer, and this second edition represents much revision of data by J. A. Riddick and E. E. Toops, Jr., of Commercial Solvents Corporation. The text is divided into sections under the headings: Classification of Solvents; Physical Properties—Discussion of Properties and Criteria for their Selection; Physical Properties—Tabulation, Tables of Physical Constants; Criteria of Purity, Drying and Determination of Water; and Purification Methods.

The extensive coverage of the text material is indicated by the 2,147 references arranged alphabetically at the back of the book. There is also a general index and cumulative author and subject indexes for Volumes I to VIII.

This volume as well as the others in this excellent series should definitely be available to graduate students and chemists in all fields.

Polarographic Techniques. By LOUIS MEITES. Interscience Publishers, Inc., New York, 1955. xiii + 317 pp. 16 x 23.5 cm. Price $6.

This book is intended to serve as an introduction to the field for students, practical analysts, and research chemists. The theoretical material included in the text was added to explain and supplement the practical applications and experimental manipulations described. Complete and detailed directions for common manipulations involved in polarographic work to enable the beginner to avoid errors and unnecessary trials are given for a number of experiments.

The text material is divided under the following section headings: The Nature and Scope of Polarographic Measurements; Instrumentation; The Polarographic Limiting Current; Theory of the Current-Voltage Curve; The Interpretation of Half-Wave Potential Data; Maxima and Their Suppression; Techniques of Quantitative Polarographic Analysis; Amperometric Titrations; and Special Techniques. Appendixes give information on "trouble shooting in polarographic circuits" and half-wave potentials, and diffusion current constants of inorganic substances. An extensive tabulation of the potentials and constants is included along with 307 literature references. Polarographic spectra are given on the front and back lining papers of the book where they can serve as convenient references. The type and format of the book are excellent, and a subject index is included.

This book should be a useful addition to libraries available to graduate students and to control and experimental analytical laboratories.

Origins of Resistance to Toxic Agents. Proceedings of the symposium held in Washington, D. C., March 25–27, 1954. M. G. SEVAG, ROGER D. REID, and ORR E. REYNOLDS, Editors. Academic Press, Inc., New York, 1955. xv + 471 pp. 16 x 23.5 cm. Price $12.

The general basis for this symposium is a thorough exploration of available knowledge to seek explanations for the development of resistance by microbial strains to sulfonamides and other antibiotics. The concept formulated in this symposium is that the development of resistance to various toxic agents is associated with alterations and/or loss in enzyme proteins. The papers present material "... capable of benefiting physicians, biochemists, pharmacologists, entomologists, plant physiologists, students of cancer, and those who are interested in the theory of the evolution of living matter." Twenty papers are given, and the resulting discussions are included. The material was presented at five sessions under the headings: resistance to microbicides; resistance to herbicides and insecticides; tolerance and addiction to drugs and alcoholism; resistance factors, infectious agents, and cancer cells; and physiological, chemical, and genetic viewpoints. General author and subject indexes are appended.

College Physiology. By DONALD M. PACE and BENJAMIN W. MCCASHLAND. Thomas Y. Crowell Co., New York, 1955. xxi + 615 pp. 16 x 23.5 cm. Price $5.50.

This excellently written and well illustrated text is intended to provide the foundation required by students interested in the various fields of zoology and it stresses particularly the functions of the human body. Discussions are clear and brief, developing the material for the beginning student. The clarity and brevity also make this a good quick reference book for basic points in physiology. The forty chapters are divided into eight parts under the headings: General; Structure and Movement; Nervous Coordination; Receptors; The Transport System; Respiration; Nutrition and Elimination; and Endocrine Glands and Reproduction. The book should be considered for use as a textbook by teachers of physiology in all college courses, including those in pharmacy schools.

Biochemistry and Physiology of Protozoa. Vol. 2. S. H. HUTNER and ANDRE LWOFF, Editors. Academic Press, Inc., New York, 1955. xiii + 388 pp. 16 x 23.5 cm. Price $9.

This second volume, appearing four years after Volume I, presents much new information in the field of protozoology. The increased knowledge of culture methods and biochemical techniques have prepared the way for a greatly widened use of protozoa as biochemical tools. Pharmacists will be particularly interested in the chapter on "The Chemotherapy of Malaria, Piroplasmosis, Trypanosomiasis, and Leishmaniasis" by L. S. Goodwin and I. M. Rollo. The book is an excellent addition in

its field, and its use as a reference is aided by the appended author and subject indexes.

Analysis of Insecticides and Acaricides. A treatise on sampling, isolation, and determination, including residue methods. By FRANCIS A. GUNTHER and ROGER C. BLINN. Interscience Publishers, Inc., New York, 1955. xi + 696 pp. 16 x 23.5 cm. Price $14.

This book which is volume VI in a series, seven volumes of which have been prepared, is an excellent presentation of the analytical methods used in the determination of residual insecticides (including acaricides) in plants, plant parts, and soils; and in the assays of insecticides in technical grade materials and formulated products.

The methods for residue analysis are particularly interesting and valuable, because many special techniques and modifications of general procedures developed and used at the University of California Citrus Experiment Station are included. The text covers the subject thoroughly, starting with a discussion of persisting residues as an analytical problem, considerations of presampling and sampling problems, preanalytical treatment of materials, the analysis of samples, and interpretation of data. The chapter on analytical procedures covers 355 pages of the text.

Detailed ultraviolet and infrared spectra for widely used insecticides and acaricides are appended, and a subject index is given. The text is well written with excellent illustrations and photographs included. The book should be a must for analytical libraries; and teachers will find many helpful discussions of practical applications of special procedures.

Vitamins in Theory and Practice. By L. J. HARRIS. Cambridge University Press, Cambridge, England, and New York, 1955. 22 + 366 pp. 16 x 21 cm. Price $6.50.

This British book aims to present in a readable manner to the layman many facts about vitamins. It is noted that a round score of vitamins is discussed in this fourth edition, as compared with the bare half-dozen known when the first edition appeared in 1935. The material included is presented in an easy style with many pictures and tabulations to make an interesting book.

The Medical Clinics of North America. Symposium from New York on Basic Sciences in Medical Practice with 17 Clinics. W. B. Saunders Company, Philadelphia, 1955. viii + 300 pp. 15.5 x 23 cm.

The May issue is the third of the 1955 series, the other two were in January and March. The subjects covered by authoritative writers in this issue are: fluids and electrolytes; cardiac physiology and circulatory hemodynamics; pulmonary function in health and disease; renal function in health and disease; hepatic function and protein metabolism in cirrhosis of the liver; the anemias; the allergic reaction; basic sciences in relation to rheumatic diseases; stress and adaptive patterns resulting in tissue damage in man; some clinical aspects of thyroid physiology; adrenal cortical physiology in health and disease; carbohydrate metabolism; fat metabolism, obesity and hypercholesterolemia; protein metabolism; calcium metabolism in health and disease; and antibiotics.

These reports cover current literature and should be very helpful to the physician who does not have time to review many medical journals.

Analytical Cytology. ROBERT C. MELLORS, Editor. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. 16 x 23.5 cm. Price $15.

This book attempts rather successfully to bring together some of the applications of physics and chemistry to the analysis of cellular structure and function. The text material contributed by a number of authors is presented under the following chapter headings: Cytophotometric Analysis in the Visible Spectrum; Histochemical and Cytochemical Staining Methods; Phase-contrast, Interference-contrast, and Polarizing Microscopy; Ultraviolet Microscopy and Microspectroscopy; Fluorescence Microscopy; The Electron Microscopy of Tissue Cells; Radioautography, Its Use in Cytology; Historadiography; and X-ray Diffraction Techniques and Their Application to the Study of Biomolecular Structures.

The first chapter includes interesting information on the comparison of cytophotometry with cytology, its relation with histochemistry; and a concise practical and theoretical review of instrumentation with illustrations. Diagrams and photographs are used effectively throughout the text. References are given at the end of each chapter and a general index is appended. The book should be valuable to those interested in the field of cellular form and function.

New and Nonofficial Remedies, 1955. The Council on Pharmacy and Chemistry, American Medical Association. J. B. Lippincott Company, Philadelphia, 1955. xlviii + 653 pp. 13.5 x 18.3 cm. Price $3.35.

N. N. R., 1955, is the latest edition of this valuable publication of the Council of Pharmacy and Chemistry of the American Medical Association. Included in the 1955 edition are articles, including 42 new ones, that the Council found acceptable under its former rules through the period ending January 1, 1955. Future editions of N. N. R. will be prepared according to new regulations as announced in *J. Am. Med. Assoc.*, Feb. 19, 1955.

In addition to revisions in the general statements and individual monographs and the new additions, 14 N. N. R., 1954, monographs, 10 of which are covered by the U. S. P., have been dropped. Eight other drugs were dropped because the manufacturers of the only Council-accepted dosage forms discontinued their manufacture. The "Tests and Standards for N. N. R." continues to be published as a separate volume. Since the 1953 edition, the bibliography of unaccepted products has been omitted. The index to distributors and the general index are included.

N. N. R. continues to be a reference volume that all pharmacy libraries must have.

The Pharmacopeia of the United States of America. 15th rev. By authority of The United States Pharmacopoeial Convention, Inc. meeting at Washington, D. C., May 9 and 10, 1950. Prepared by the Committee of Revision and published by the Board of Trustees. Mack Publishing Company, Easton, Pa., 1955. Lii + 1178 pp. 15.5 x 23.5 cm. Price $10, domestic and $10.50, foreign.

In this era of rapidly changing materia medica, an extensive revision of a complex book of standards such as the U. S. P. is a task, the enormity of which is difficult to comprehend unless one has participated in this or similar work. Dr. Lloyd C. Miller, Director of Pharmacopeial Revision, and those who aided him have performed their tasks well.

The Constitution and By-laws of the U. S. Pharmacopoeial Convention were revised at the 1950 meeting and the entire adopted text and an abstract of the proceedings of the 1950 meeting of the U. S. P. Convention are included in the "front" material of U. S. P. XV. Very few persons will miss the omitted list of English-Spanish titles of U. S. P. articles that has heretofore appeared in this section of the U. S. P.

The monograph section of U. S. P. XV includes, 838 items. Newly admitted are 242 monographs, while 160 monographs that appeared in U. S. P. XIV have been dropped.

Inclusion of more information in the monographs, particularly the detailed procedures for testing antibiotics and the biologics, has increased the average length of the monographs. Practically all the information available on the tests and standards for biological products and all of the tests of the federal regulations on the antibiotics are included.

The following changes are noted in light principal categories of drugs in U. S. P. XIV and U. S. P. XV, respectively: antihistaminics, 2 to 13; antibiotics, 5 to 12; diagnostic aids, 9 to 16; analgetics and sedatives, 18 to 21; biologics, 29 to 34; chemotherapeutic agents, 27 to 31; endocrine preparation (including corticosteroids), 18 to 25; and vitamins, 13 to 15.

Included among the seven new diagnostic aids is sodium radioiodide solution which is used for diagnosis of thyroid disorders and is the first radioactive drug to be given Pharmacopeial status. Of the 18 monographs on antibiotics in U. S. P. XIV, only five were retained so that 37 of the 42 U. S. P. XV monographs on antibiotics are new.

Of particular interest is the addition in each monograph of a "category" classification indicating pharmacologic or therapeutic action or other use of the item. A dosage range as well as the usual dose is included wherever feasible.

The section on General Tests has been revised and greatly expanded in content while holding down the relative number of pages occupied by using a brief style of presentation. New chapters cover chromatography, pharmaceutical preparations, radioactivity, titrimetry, biological reagents for clinical tests, microbial assays of antibiotics, and a comprehensive and detailed chapter on the design and analysis of biological assays. New tests include one on pressor substances, one for biological adequacy of protein, tests for transfusion and infusion assemblies, a solubility test for hypodermic tablets, a disintegration test for coated tablets, and a limit on the weight variation of capsule contents. A chapter on sterilization has been included after having been omitted from U. S. P. XIV.

The valuable tabulated information that has appeared in the "back" part of earlier revisions has been brought up to date for U. S. P. XV.

The printing style and format have not been changed but, in order to keep the bulk of U. S. P. XV (which has 120 pages more than U. S. P. XIV) within reasonable limits, paper of lighter stock has been used. All who obtain copies of U. S. P. XV will find it a valuable reference volume in addition to its necessity as a legal compendium.

Methods for Evaluation of Nutritional Adequacy and Status. A Symposium sponsored by the Quartermaster Food and Container Institute, Washington, 1954.

The objectives of the symposium recorded in this 313-page compilation were: to review critically the merits of existing methods of evaluating nutritional adequacy, to emphasize the needs for special methods not now available, and to consider suggestions for developing special methods. Papers and discussions are grouped under the headings: introduction, evaluation of protein, of vitamin, and of mineral adequacy; evaluation of military rations by animal experimentation; evaluation of nutritional status of populations; discussion on body composition. Indexes to the interesting material covered in such symposia are very helpful and interested readers would prefer to pay enough for such reports to cover the cost of indexing.

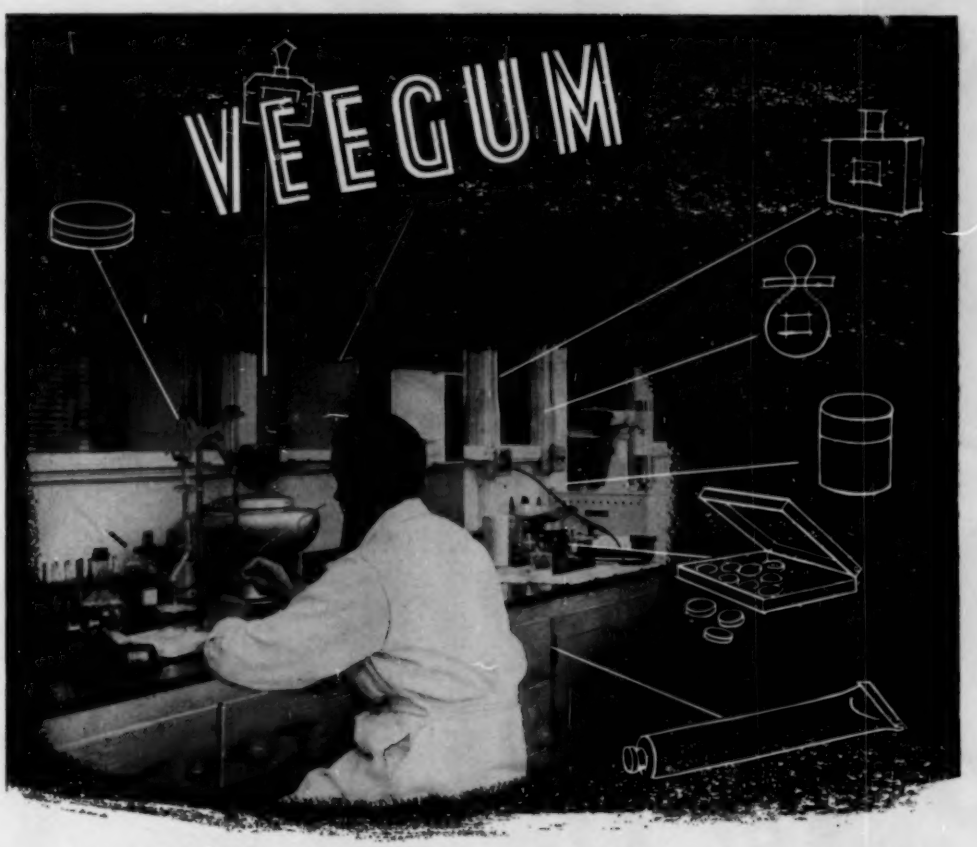
Amphetamine in Clinical Medicine: Actions and Uses. By W. R. BETT, LEONARD H. HOWELLS, and A. D. MACDONALD. The Williams & Wilkins Co., Baltimore, 1955. (E. & S. Livingstone, Ltd., Edinburgh and London). 78 pp. 13 x 19 cm. Price $2.75.

This pocket-size book is not presented as a comprehensive review but is intended as a guide to *when* and *when not* to employ the useful but often misused amphetamines. The discussions under pharmacology, overweight, fatigue and depression, narcolepsy, alcoholism and other drug addictions, barbiturate intoxication, psychopathic states, enuresis and other behavior disorders in children are directed to the physician but should interest pharmacists as well.

A Dictionary of Terms in Pharmacognosy and Other Divisions of Economic Botany. By GEORGE N. HOCKING. Charles C Thomas, Springfield, Ill. 1955. xxv + 284 pp. 16.5 x 24.5 cm.

This dictionary is designed to meet the special needs of students and practitioners in the various health professions, of people in trade and industry, and of others interested in learning more about the useful plants and animals. If it is possible to meet these needs, Dr. Hocking has done it. Valuable additional reference material is included in appendixes. Appendix D is a list of terms describing properties and therapeutic uses of drugs.

This book should be available to anyone who might seek information in the medical care field and particularly to pharmacists, pharmacy students, and their instructors.



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